JC02 Rec'd PCT/PTO 01 APR 2002

TRANSMITTAL LETTER OT THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	Attorney Docket No. **0512-1030** U.S. Application No. **10/089612**

INTERNATIONAL APPLN. NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR00/02596	**19 SEPTEMBER 2000**	**1 OCTOBER 1999**

TITLE OF INVENTION: **PLANT SEED ENDOSPERM-SPECIFIC PROMOTERS**

APPLICANT(S) FOR DE/EO/US: **JEAN-FRANCOIS BONELLO, PETER ROGOWSKY, PASCUAL PEREZ**

Applicant herewith submits to the United States Designated Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c)(2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau)
   b. ☐ has been communicated by the International Bureau. See attached PCT/IB/308.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371 (c)(2))
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made, however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☒ Information Disclosure Statement (IDS) w/PTO-1449 - ☒ Copy of IDS citations
12. ☐ Assignment Papers (cover sheet & document(s))
13. ☒ A FIRST Preliminary Amendment.
14. ☐ A SECOND or SUBSEQUENT Preliminary Amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application (35 U.S.C. 154(d)(4)).
20. ☒ Other items or information: **International Search Report, PCT/IB/304, PCT/IPEA/409, Abstract of the Disclosure on a Separate Sheet, Application Data Sheet**

JC10 Rec'd PCT/PTO 01 APR 2002

U.S. APPLICATION NO. 10/089612	INTERNATIONAL APPLN. NO. PCT/FR00/02596	ATTORNEY DOCKET NO. 0512-1030

21. ☒ The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1)-(5):		
Neither international preliminary examination fee nor international search fee paid to USPTO and international Search Report not prepared by the EPO or JPO $1040.00		
International preliminary examination fee not paid to USPTO but International Search Report prepared by the EPO or JPO $890.00		
International preliminary examination fee not paid to USPTO but International search fee paid to USPTO $740.00		
International preliminary examination fee paid to USPTO but all claims did not satisfy provision of PCT Article 33 (1)-(4) $710.00		
International preliminary examination fee paid to USPTO and all claims satisfied provision of PCT Article 33 (1)-(4) $100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT	$ 890.00	
Surcharge of $130.00 for furnishing the oath or declaration later than ☐ 20- ☒ 30 months from the earliest claimed priority date (37 CFR 1.492(e))	$ 130.00	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	29 - 20 =	9	X $18.00	$ 162.00	
Independent Claims	20 - 3 =	17	X $84.00	$ 1,428.00	
MULTIPLE DEPEND CLAIM(S) (if applicable)			+ $280.00	$	
			TOTAL OF ABOVE CALCULATION -	$ **2,480.00**	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by ½.			+	$	
			SUBTOTAL =	$ 2,480.00	
Processing fee of $130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492Z(f)).				$	
			TOTAL NATIONAL FEE =	$ 2,480.00	
Fee for recording the enclosed assigned (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) $40.00 per property +				$	
			TOTAL FEES ENCLOSED -	$ 2,480.00	
				Amount to be refunded:	$
				Charged:	$

☒ A Check in the amount of **$2,480.00** to cover all fees is attached.

☐ The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to Deposit account No. 25-0120 in the name of Young & Thompson, as described below. A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 25-0120 for any additional fee required under 37 C.F.R. §§ 1.16 or 1.17.

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00466
PATENT TRADEMARK OFFICE

BC/ia
Date: **April 1, 2002**

SIGNATURE Benoit Castel

Benoit Castel
NAME

35,041
REGISTRATION NO.

Application Data Sheet

Application Information

Application Type::	Regular
Subject Matter::	Utility
Suggested Classification::	
Suggested Group Art Unit::	
CD-ROM or CD-R?::	None
Number of CD disks::	
Number of Copies of CDs::	
Sequence Submission?::	None
Computer Readable Form (CRF)::	No
Number of copies of CRF::	0
Title::	PLANT SEED ENDOSPERM-SPECIFIC PROMOTERS
Attorney Docket Number::	0512-1030
Request for Early Publication?::	No
Request for Non-Publication?::	No
Suggested Drawing Figure::	
Total Drawing Sheets::	11
Small Entity?::	No
Latin Name::	
Variety Denomination Name::	
Petition Included?::	No
Petition Type::	
Licensed US Gov't Agency::	
Contract or Grant Numbers::	
Secrecy Order in Parent Appl.?::	No

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Correspondence Information

Correspondence Customer Number:: 000466

Representative Information

Representative Customer Number::	000466

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This application	National Stage of	PCT/FR00/02596	9/19/00

Foreign Priority Information

Country::	Application Number::	Filing Date::	Priority Claimed::
FRANCE	99 12305	10/1/99	Yes

Assignment Information

Assignee Name::

Street of Mailing Address::

City of Mailing Address::

State or Province of Mailing Address::

Country of Mailing Address::

Postal or Zip Code of Mailing Address::

PATENT
0512-1030

IN THE U.S. PATENT AND TRADEMARK OFFICE

In re application of: Jean-Francois BONELLO et al.

Appl. No.: **NEW** Group:

Filed: April 1, 2002 Examiner:

For: PLANT SEED ENDOSPERM-SPECIFIC PROMOTERS

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, DC 20231

April 1, 2002

Sir:

The following preliminary amendments and remarks are respectfully submitted in connection with the above-identified application.

IN THE CLAIMS:

Please cancel claims 1-16 without prejudice or disclaimer of the subject matter contained therein.

Please add the following claims:

--17. (new) An isolated promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of an endosperm

surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm.--

--18. (new) A promoter nucleotide sequence according to claim 17, isolated from cereals.--

--19. (new) A promoter nucleotide sequence according to claim 17, isolated from maize.--

--20. (new) A promoter nucleotide sequence according to claim 17, comprising a sequence chosen from amongst the sequences SEQ ID N° 1, N° 2, N° 3, N° 4, N° 5, N° 6 and N° 7, and any sequence which is a homologue of these.--

--21. (new) A promoter nucleotide sequence according to claim 17, also comprising a regulator element in *cis* defined by the pattern CTACACCA.--

--22. (new) A promoter nucleotide sequence according to claim 17, also comprising a regulator element in *cis* defined by the pattern CTACACCA, repeated in tandem.--

--23. (new) An expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of the endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest.--

--24. (new) An expression cassette according to claim 23, in which the gene of interest codes for a protein is chosen from amongst a protein involved in the biological characteristics selected from the group consisting of the development of the embryo, the development of the endosperm, the cell growth, the metabolism of sugars, the metabolism of the fatty acids, the metabolism of a toxic protein and the metabolism of a transcription inhibiting protein.--

--25. (new) An expression cassette according to claim 23, in which the gene of interest codes for a first protein is chosen from amongst barnase and isopentenyltransferase.--

--26. (new) An expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest.--

--27. (new) An angiosperm plant host cell, transformed by an expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular

in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest.--

--28. (new) An angiosperm plant host cell in the form of cereal transformed by an expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest.--

--29. (new) A transgenic plant, selected from the group consisting of fruit, seed, grain and pollen, generated from an angiosperm plant host cell, transformed by an expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest.--

--30. (new) A part of a transgenic plant, selected from the group consisting of fruit, seed, grain and pollen, generated from

an angiosperm plant host cell, transformed by an expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest.--

--31. (new) A transgenic plant, generated from an angiosperm plant host cell, transformed by an expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest, wherein the transgenic plant is a plant selected from the group consisting of a cereal, an oily plant, maize, wheat, rape and sunflower.--

--32. (new) A part of a transgenic plant selected from the group consisting of fruit, seed, grain and pollen, generated from an angiosperm plant host cell, transformed by an expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding

sequences to which it is bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest, wherein the transgenic plant is a plant selected from the group consisting of a cereal, an oily plant, maize, wheat, rape and sunflower.--

--33. (new) A hybrid transgenic plant obtained by crossing parts of transgenic plants, each part of the transgenic plant being selected from the group consisting of fruit, seed, grain and pollen, generated from an angiosperm plant host cell, transformed by an expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest.--

--34. (new) A hybrid transgenic plant obtained by crossing transgenic plants, generated from an angiosperm plant host cell, transformed by an expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is

bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest, wherein the transgenic plant is a plant selected from the group selected from a cereal, an oily plant, maize, wheat, rape and sunflower.--

--35. (new) A method of obtaining an angiosperm plant having improved agronomic or nutritional qualities, comprising the steps consisting of:

- transforming at least one angiosperm plant cell by means of an expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest; and

- cultivating the cell thus transformed so as to generate a plant containing in its genome an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression

being i) specific to the region of the endosperm surrounding the embryo in the seeds of the angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest.--

--36. (new) Use of an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest, said use of the expression cassette being for obtaining a transgenic angiosperm plant exhibiting improved agronomic qualities.--

--37. (new) Use of an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest, said use of the expression cassette being for obtaining a transgenic angiosperm plant exhibiting improved nutritional qualities.--

--38. (new) Use according to claim 36, for obtaining a transgenic plant producing seeds selected from the group consisting of starch contents modified compared with a non-transformed plant.--

--39. (new) Use according to claim 36, for obtaining a transgenic plant producing seeds selected from the group consisting of oil contents modified compared with a non-transformed plant.--

--40. (new) Use of a plant, selected from the group consisting of a seed, grain and fruit, generated from an angiosperm plant host cell, transformed by an expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest, the plant being for the preparation of derived products, notably food products.--

--41. (new) Use of a plant, selected from the group consisting of a seed, grain and fruit, generated from an angiosperm plant host cell, transformed by an expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i)

specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest, the part of the plant being for the preparation of derived products, notably food products.--

--42. (new) Use of a transgenic plant, generated from an angiosperm plant host cell, transformed by an expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest, wherein the transgenic plant is a plant selected from the group consisting of a cereal, an oily plant, maize, wheat, rape and sunflower, the plant being for the preparation of derived products, notably food products.--

--43. (new) Use of a part of a transgenic plant, selected from the group comprising a seed, grain and fruit, generated from an angiosperm plant host cell, transformed by an expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i)

specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest, the plant being for the preparation of derived products, notably food products.--

--44. (new) Use of a part of a hybrid transgenic plant, selected from the group comprising a seed, grain and fruit, generated from an angiosperm plant host cell, transformed by an expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest, the plant being for the preparation of derived products, notably food products.--

--45. (new) Use of a hybrid transgenic plant, generated from an angiosperm plant host cell, transformed by an expression vector containing an expression cassette comprising an isolator promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, the said expression being i) specific to the region of an endosperm surrounding an embryo in seeds of angiosperms and ii) intervening in particular in the

early stages of development of the endosperm, the expression cassette being effectively bound to at least one gene of interest, wherein the hybrid transgenic plant is a plant selected from the group comprising a cereal, an oily plant, maize, wheat, rape and sunflower, the plant being for the preparation of derived products, notably food products.--

Docket No. 0512-1030

REMARKS

Claims 17-45 are pending in the present application. Claims 1-16 have been cancelled and claims 17-45 have been added.

Entry of the above amendments is earnestly solicited. An early and favorable first action on the merits is earnestly requested.

Should there be any matters that need to be resolved in the present application, the Examiner is respectfully requested to contact the undersigned at the telephone number listed below.

The Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 25-0120 for any additional fees required under 37 C.F.R. § 1.16 or under 37 C.F.R. § 1.17.

Respectfully submitted,

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Attachments

Atty. Docket No. 0512-1030

PATENTS

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Jean-Francois BONELLO et al. Confirmation No. 2604

Serial No. 10/089,612 BOX PCT

Filed April 1, 2002

PLANT SEED ENDOSPERM-SPECIFIC PROMOTERS

PRELIMINARY AMENDMENT

Commissioner for Patents

Washington, D.C. 20231

Sir:

Prior to the first Official Action, please amend the above-identified application as follows:

IN THE SPECIFICATION:

Replace the paragraph beginning at page 2, line 29, with the following rewritten paragraph:

--The promoter sequences of invention can advantageously be selected from the group consisting of the sequence comprising the sequences SEQ ID NO: 1, NO: 2, NO: 3, NO: 4, NO: 5, NO: 6 or NO: 7 and any nucleotide sequence which is a homologue of these.--

Replace the paragraph beginning at page 3, line 1, with the following rewritten paragraph:

--The sequence SEQ ID NO: 1 corresponds to the gene promoter *Esr1*.--

Replace the paragraph beginning at page 3, line 5, with the following rewritten paragraph:

--The sequence SEQ ID NO: 2 corresponds to the gene promoter *Esr2*.--

Replace the paragraph beginning at page 3, line 8, with the following rewritten paragraph:

--The sequence SEQ ID NO: 3 corresponds to the gene promoter *Esr*3.--

Replace the paragraph beginning at page 3, line 11, with the following rewritten paragraph:

--The sequence SEQ ID NO: 4 corresponds to the gene promoter *Esr*4.--

Replace the paragraph beginning at page 3, line 14, with the following rewritten paragraph:

--The sequence SEQ ID NO: 5 corresponds to a fragment of 499 pairs of bases on SEQ ID NO: 2 (nucleotides 1995-2493).--

Replace the paragraph beginning at page 3, line 17, with the following rewritten paragraph:

--The sequence SEQ ID NO: 6 corresponds to a fragment of 507 pairs of bases on SEQ ID NO: 3 (nucleotides 1202-1708).--

Replace the paragraph beginning at page 3, line 20, with the following rewritten paragraph:

--The sequence SEQ ID NO: 7 is a consensus sequence of 265 nucleotides, obtained by means of comparison between the sequences SEQ ID NO: 1, NO: 2 and NO: 3.--

Replace the paragraph beginning at page 3, line 24, with the following rewritten paragraph:

--"Homologous nucleotide sequence" means any nucleotide sequence which differs from the sequence SEQ ID NO: 1, NO: 2, NO: 3, NO: 4, NO: 5, NO: 6 or NO: 7, by a substitution, deletion and/or insertion of one or more nucleotides, at positions such that these homologous nucleotide sequences preserve the property of specific promoter of the sequences SEQ ID NO: 1 to NO: 7.--

Replace the paragraph beginning at page 3, line 32, with the following rewritten paragraph:

--Preferably such a homologous nucleotide sequence is identical to at least 70% of the sequences SEQ ID NO: 1 to NO: 7, preferably at least 80%, preferably still at least 95%.--

Replace the paragraph beginning at page 4, line 18, with the following rewritten paragraph:

--Preferentially, such homologous nucleotide sequences specifically hybridises to the sequences which are complementary to the sequences SEQ ID NO: 1 to NO: 7 under stringent conditions. The parameters defining the stringency conditions depend on the temperature at which 50% of the paired strands separate (Tm).--

Replace the paragraph beginning at page 5, line 9, with the following rewritten paragraph:

--The various nucleotide sequences of the invention can be of artificial origin or not. They may be DNA sequences

obtained by sieving banks of sequences by means of sensors produced on the basis of the SEQ ID NO: 1 to NO: 7. Such banks can be prepared by conventional techniques of molecular biology, known to persons skilled in the art.--

Replace the paragraph beginning at page 13, line 9, with the following rewritten paragraph:

--Figure 9 shows a comparison of the sequences of the promoters of the genes *Esr1, Esr2 and Esr3* (respectively prEsr1, prEsr2 and prEsr3, or residues 271 to 531 of SEQ ID NO: 1, residues 1992 to 2493 of SEQ ID NO: 2 and residues 1199 to 1708 of SEQ ID NO: 3), the preserved parts being aligned.--

Replace the paragraph beginning at page 14, line 25, with the following rewritten paragraph:

--As illustrated in Figure 1, fragments containing the open reading phases of Esr1, Esr2 and Esr3 (Opsahl et al., 1997) as well as upstream and downstream sequences were sub-cloned in the plasmid pBluescript SK+ (Stratagene) in accordance with the methods described in Sambrook et al. (1989). The result was the plasmid L23/7 containing a fragment SalI of 2.1 kb of λEsr1g1 and the plasmid L33/1 containing a fragment BamHI of 3.4 kb of λEsr2g1. The plasmid L33/10 containing a fragment BamHI of 1.9 kb of λEsr2g1 and the plasmid L102c24 containing a fragment HindIII of 4.5 kb of λE1-111. XbaI sites situated just upstream of the open reading phase (TCTAGATTCCATG) (SEQ ID NO: 22) made it

possible to differentiate the putative promoters of the respective open reading phases. In particular, the fragment SaII/XbaI of 0.53 kb of L23/7 was designated as a putative promoter of Esr1, the fragment HindIII/BamHI of 2.35 kb of L33/1 (upstream part of the promoter) and the fragment BamHI/XbaI of 0.14 kb of L33/10 (downstream part of the promoter), that of the putative promoter of Esr2, the fragment HindIII/XbaI of 1.71 kb, that of the putative promoter of Esr3 and the fragment XbaI/XbaI of 1.62 kb comprising the putative promoter of Esr4. A functional promoter Esr2 of 2.49 kb was reconstructed from the fragment HindIII/BamHI of 2.35 kb of L33/1 and from the fragment BamHI/XbaI of 0.14 kb of L33/10, in a base plasmid of the pBSSK+ type (Stratagene). The orientation of the arrows in Figure 1 represents the orientation 5'-3'.--

Replace the paragraph beginning at page 16, line 4, with the following rewritten paragraph:

--The consensus sequence (SEQ ID NO: 7) was obtained after alignment of the three promoter nucleotide sequences and using Sequencher 3.1 software from Genes Codes Corporation (Ann Arbor, MI 48106).--

Replace the paragraph beginning at page 17, line 8, with the following rewritten paragraph:

--A homology is also observed between the proximal regions which extend over approximately 500 pairs of bases between the promoter of *Esr2* and that of *Esr3*, as defined by the

sequences SEQ ID NO: 5 and NO: 6.--

Replace the paragraph beginning at page 19, line 5, with the following rewritten paragraph:

--* the numbering of the promoter Esr2 is based on the sequence pEsr2 presented in an annexe (SEQ ID NO: 2) which goes from HindIII (AAGCTT or A = position 1) to XbaI (TCTAGA or A = position 2493).--

Replace the paragraph beginning at page 20, line 1, with the following rewritten paragraph:

--Secondly, fragments of the promoter were amplified using the initiator ESRX (5'GGGGTCTAGACTGTGAAGCTATTTTCCA3' (SEQ ID NO: 8)) containing the restriction site XbaI (underlined) and ESRH1 (5'GGGGAAGCTTTACATTCTTGCCATAACATA3' (SEQ ID NO: 9)), ESRH2 (5'GGGGAAGCTTTTCATCAATAATGCCTCATT3' (SEQ ID NO: 10)) or ESRH3 (5'GGGGAAGCTTTAATTTCTTACTTCCTATCT3' (SEQ ID NO: 11)) containing the HindIII restriction site (underlined). The amplification products digested by XbaI and HindIII replaced the entire promoter *Esr2* upstream of the gene of the β-glucuronidase in the plasmid L124/19.--

Replace the paragraph beginning at page 20, line 13, with the following rewritten paragraph:

--The deleted promoters associated with the β-glucuronidase gene were then cloned in a plasmid containing the luciferase gene under the control of the promoter of the rice actin. The latter was obtained by cloning the fragment XhoI/NcoI

of the plasmid pAct1-F4 (Mc Elroy D. et al., Mol Gen Genet., 231: 150-160, 1991) corresponding to the promoter and first intron of the rice actin, in a plasmid of the pGP214 type containing the luciferase gene and the terminator of nopaline synthase (Twell D. et al., Development 109, 705-713, 1990), digested by SalI/NcoI. An adaptor containing the restriction sites SalI and NotI, formed in nucleotides 5'GGCCAGTCGACAAAGCGGCCGCATGCA3' (SEQ ID NO: 12) and 5'TCAGCTGTTTCGCCGGCGT3' (SEQ ID NO: 13) was introduced into the plasmid obtained, digested by NotI and PstI (plasmid L210). The fragments SalI/NotI containing the deleted promoters associated with the β-glucuronidase gene were cloned in the plasmid L210 digested by SalI and NotI.--

Replace the paragraph beginning at page 23, line 8, with the following rewritten paragraph:

--[1)] adaptor JFB34:5'TCGACTGCAGCCCA 3' (SEQ ID NO: 14)

3'GACGTCGGGTTCGA 5' (SEQ ID NO: 15)

[2)] adaptor JFB56: 5'CTAGACCCGAATTCGC 3' (SEQ ID NO: 16)

3'TGGGCTTAAGCGCCGG 5' (SEQ ID NO: 17)--

Replace the paragraph beginning at page 27, line 19, with the following rewritten paragraph:

--In a plasmid derived from pJIT30 containing the promoter *35S*, a multiple cloning site and the terminating sequence of the cabbage mosaic virus (Guerineau F. et al., Plant Mol Biol, 15: 127-136, 1990), an adaptor containing a SpeI site and formed by the oligonucleotides (5'GATCCACTAGTCCCG (SEQ ID NO:

18)) and (5'AATTCGGGACTAGTG (SEQ ID NO: 19)) was inserted between the sites BamHI and EcoRI. The fragment EcoRI/SpeI of the plasmid L42 a14 (Opsahl-Ferstad et coll., 1997) was inserted in the plasmid described previously. The construction thus obtained contained the gene *Esr2* in antisense orientation under the control of the promoter 35S (plasmid L79 b5).--

Replace the paragraph beginning at page 28, line 8, with the following rewritten paragraph:

--The promoter *35S* was eliminated in the plasmid L79 b5 by restriction by SacI and HindIII, and replaced by an adaptor containing the restriction site HindIII and NotI and formed by the oligonucleotides (5'AAGCTTTTTGCGGCCGC (SEQ ID NO: 20)) and (5'TCGAGCGGCCGCAAAAAGCTTAGCT (SEQ ID NO: 21)). The promoter *Esr2* in the form of a fragment HindIII/NotI of 2.44 kb was introduced into this adaptor. The construction thus obtained contains the gene *Esr2* in antisense orientation under the control of its own promoter (plasmid L129/46 (cf Figure 11)).--

IN THE CLAIMS:

Amend claim 20 as follows:

20. (amended) A promoter nucleotide sequence according to claim 17, comprising a sequence chosen from amongst the sequences SEQ ID NO: 1, NO: 2, NO: 3, NO: 4, NO: 5, NO: 6 and NO: 7, and any sequence which is a homologue of these.

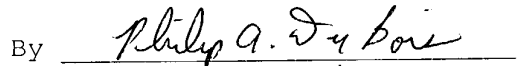
REMARKS

The above changes in the specification and claims amend the present application so that it is commensurate in scope with the present Sequence Listing.

Attached hereto is a marked-up version of the changes made to the specification and claims. The attached page is captioned "VERSION WITH MARKINGS TO SHOW CHANGES MADE."

Respectfully submitted,

YOUNG & THOMPSON

By 

Philip A. DuBois
Agent for Applicants
Registration No. 50,696
745 South 23rd Street
Arlington, VA 22202
Telephone: 521-2297

September 18, 2002

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

Paragraph beginning at page 2, line 29, has been amended as follows:

The promoter sequences of invention can advantageously be selected from the group consisting of the sequence comprising the sequences SEQ ID [N° 1, N° 2, N° 3, N° 4, N° 5, N° 6 or N° 7] NO: 1, NO: 2, NO: 3, NO: 4, NO: 5, NO: 6 or NO: 7 and any nucleotide sequence which is a homologue of these.

Paragraph beginning at page 3, line 1, has been amended as follows:

The sequence SEQ ID [N°] NO: 1 corresponds to the gene promoter *Esr1*.

Paragraph beginning at page 3, line 5, has been amended as follows:

The sequence SEQ ID [N°] NO: 2 corresponds to the gene promoter *Esr2*.

Paragraph beginning at page 3, line 8, has been amended as follows:

The sequence SEQ ID [N°] NO: 3 corresponds to the gene promoter *Esr3*.

Paragraph beginning at page 3, line 11, has been amended as follows:

The sequence SEQ ID [N°] NO: 4 corresponds to the gene

promoter *Esr*4.

Paragraph beginning at page 3, line 14, has been amended as follows:

The sequence SEQ ID [N°] NO: 5 corresponds to a fragment of 499 pairs of bases on SEQ ID [N°] NO: 2 (nucleotides 1995-2493).

Paragraph beginning at page 3, line 17, has been amended as follows:

The sequence SEQ ID [N°] NO: 6 corresponds to a fragment of 507 pairs of bases on SEQ ID [N°] NO: 3 (nucleotides 1202-1708).

Paragraph beginning at page 3, line 20, has been amended as follows:

The sequence SEQ ID [N°] NO: 7 is a consensus sequence of 265 nucleotides, obtained by means of comparison between the sequences SEQ ID [N° 1, N° 2 and N° 3] NO: 1, NO: 2 and NO: 3.

Paragraph beginning at page 3, line 24, has been amended as follows:

"Homologous nucleotide sequence" means any nucleotide sequence which differs from the sequence SEQ ID [N° 1, N° 2, N° 3, N° 4, N° 5, N° 6 or N° 7] NO: 1, NO: 2, NO: 3, NO: 4, NO: 5, NO: 6 or NO: 7, by a substitution, deletion and/or insertion of one or more nucleotides, at positions such that these homologous nucleotide sequences preserve the property of specific promoter of the sequences SEQ ID [N° 1 to N° 7] NO: 1 to NO: 7.

Paragraph beginning at page 3, line 32, has been amended as follows:

Preferably such a homologous nucleotide sequence is identical to at least 70% of the sequences SEQ ID [N° 1 to N° 7] NO: 1 to NO: 7, preferably at least 80%, preferably still at least 95%.

Paragraph beginning at page 4, line 18, has been amended as follows:

Preferentially, such homologous nucleotide sequences specifically hybridises to the sequences which are complementary to the sequences SEQ ID [N° 1 to N° 7] NO: 1 to NO: 7 under stringent conditions. The parameters defining the stringency conditions depend on the temperature at which 50% of the paired strands separate (Tm).

Paragraph beginning at page 5, line 9, has been amended as follows:

The various nucleotide sequences of the invention can be of artificial origin or not. They may be DNA sequences obtained by sieving banks of sequences by means of sensors produced on the basis of the SEQ ID [N° 1 to N° 7] NO: 1 to NO: 7. Such banks can be prepared by conventional techniques of molecular biology, known to persons skilled in the art.

Paragraph beginning at page 13, line 9, has been amended as follows:

Figure 9 shows a comparison of the sequences of the

promoters of the genes *Esr1, Esr2 and Esr3* (respectively prEsr1, prEsr2 and prEsr3, or [SEQ ID N° 1, SEQ ID N° 2, and SEQ ID N° 3] residues 271 to 531 of SEQ ID NO: 1, residues 1992 to 2493 of SEQ ID NO: 2 and residues 1199 to 1708 of SEQ ID NO: 3), the preserved parts being aligned.

Paragraph beginning at page 14, line 25, has been amended as follows:

As illustrated in Figure 1, fragments containing the open reading phases of Esr1, Esr2 and Esr3 (Opsahl et al., 1997) as well as upstream and downstream sequences were sub-cloned in the plasmid pBluescript SK+ (Stratagene) in accordance with the methods described in Sambrook et al. (1989). The result was the plasmid L23/7 containing a fragment SalI of 2.1 kb of λEsr1g1 and the plasmid L33/1 containing a fragment BamHI of 3.4 kb of λEsr2g1. The plasmid L33/10 containing a fragment BamHI of 1.9 kb of λEsr2g1 and the plasmid L102c24 containing a fragment HindIII of 4.5 kb of λE1-111. XbaI sites situated just upstream of the open reading phase (TCTAGATTCCATG) (SEQ ID NO: 22) made it possible to differentiate the putative promoters of the respective open reading phases. In particular, the fragment SaII/XbaI of 0.53 kb of L23/7 was designated as a putative promoter of Esr1, the fragment HindIII/BamHI of 2.35 kb of L33/1 (upstream part of the promoter) and the fragment BamHI/XbaI of 0.14 kb of L33/10 (downstream part of the promoter), that of the putative promoter of Esr2, the fragment HindIII/XbaI of 1.71 kb,

that of the putative promoter of Esr3 and the fragment XbaI/XbaI of 1.62 kb comprising the putative promoter of Esr4. A functional promoter Esr2 of 2.49 kb was reconstructed from the fragment HindIII/BamHI of 2.35 kb of L33/1 and from the fragment BamHI/XbaI of 0.14 kb of L33/10, in a base plasmid of the pBSSK+ type (Stratagene). The orientation of the arrows in Figure 1 represents the orientation 5'-3'.

Paragraph beginning at page 16, line 4, has been amended as follows:

The consensus sequence (SEQ ID [N°] NO: 7) was obtained after alignment of the three promoter nucleotide sequences and using Sequencher 3.1 software from Genes Codes Corporation (Ann Arbor, MI 48106).

Paragraph beginning at page 17, line 8, has been amended as follows:

A homology is also observed between the proximal regions which extend over approximately 500 pairs of bases between the promoter of *Esr2* and that of *Esr3*, as defined by the sequences SEQ ID [N° 5 and N° 6] NO: 5 and NO: 6.

Paragraph beginning at page 19, line 5, has been amended as follows:

* the numbering of the promoter Esr2 is based on the sequence pEsr2 presented in an annexe (SEQ ID [N°] NO: 2) which goes from Hindlll (AAGCTT or A = position 1) to Xbal (TCTAGA or A = position 2493).

Paragraph beginning at page 20, line 1, has been amended as follows:

Secondly, fragments of the promoter were amplified using the initiator ESRX (5'GGGGTCTAGACTGTGAAGCTATTTTCCA3' (SEQ ID [N°] NO: 8)) containing the restriction site XbaI (underlined) and ESRH1 (5'GGGGAAGCTTTACATTCTTGCCATAACATA3' (SEQ ID [N°] NO: 9)), ESRH2 (5'GGGGAAGCTTTTCATCAATAATGCCTCATT3' (SEQ ID [N°] NO: 10)) or ESRH3 (5'GGGGAAGCTTTAATTTCTTACTTCCTATCT3' (SEQ ID [N°] NO: 11)) containing the HindIII restriction site (underlined). The amplification products digested by XbaI and HindIII replaced the entire promoter *Esr2* upstream of the gene of the β-glucuronidase in the plasmid L124/19.

Paragraph beginning at page 20, line 13, has been amended as follows:

The deleted promoters associated with the β-glucuronidase gene were then cloned in a plasmid containing the luciferase gene under the control of the promoter of the rice actin. The latter was obtained by cloning the fragment XhoI/NcoI of the plasmid pAct1-F4 (Mc Elroy D. et al., Mol Gen Genet., 231: 150-160, 1991) corresponding to the promoter and first intron of the rice actin, in a plasmid of the pGP214 type containing the luciferase gene and the terminator of nopaline synthase (Twell D. et al., Development 109, 705-713, 1990), digested by SalI/NcoI. An adaptor containing the restriction sites SalI and NotI, formed in nucleotides 5'GGCCAGTCGACAAAGCGGCCGCATGCA3' (SEQ ID [N°] NO:

12) and 5'TCAGCTGTTTCGCCGGCGT3' (SEQ ID [N°] NO: 13) was introduced into the plasmid obtained, digested by NotI and PstI (plasmid L210). The fragments SalI/NotI containing the deleted promoters associated with the β-glucuronidase gene were cloned in the plasmid L210 digested by SalI and NotI.

Paragraph beginning at page 23, line 8, has been amended as follows:

1) adaptor JFB34:5'TCGACTGCAGCCCA 3' (SEQ ID [N°] NO: 14)

3'GACGTCGGGTTCGA 5' (SEQ ID [N°] NO: 15)

2) adaptor JFB56: 5'CTAGACCCGAATTCGC 3' (SEQ ID [N°] NO: 16)

3'TGGGCTTAAGCGCCGG 5' (SEQ ID [N°] NO: 17)

Paragraph beginning at page 27, line 19, has been amended as follows:

In a plasmid derived from pJIT30 containing the promoter *35S*, a multiple cloning site and the terminating sequence of the cabbage mosaic virus (Guerineau F. et al., Plant Mol Biol, 15: 127-136, 1990), an adaptor containing a SpeI site and formed by the oligonucleotides (5'GATCCACTAGTCCCG (SEQ ID [N°] NO: 18)) and (5'AATTCGGGACTAGTG (SEQ ID [N°] NO: 19)) was inserted between the sites BamHI and EcoRI. The fragment EcoRI/SpeI of the plasmid L42 a14 (Opsahl-Ferstad et coll., 1997) was inserted in the plasmid described previously. The construction thus obtained contained the gene *Esr2* in antisense orientation under the control of the promoter 35S (plasmid L79 b5).

Paragraph beginning at page 28, line 8, has been amended as follows:

The promoter *35S* was eliminated in the plasmid L79 b5 by restriction by SacI and HindIII, and replaced by an adaptor containing the restriction site HindIII and NotI and formed by the oligonucleotides (5'AAGCTTTTTGCGGCCGC (SEQ ID [N°] NO: 20)) and (5'TCGAGCGGCCGCAAAAAGCTTAGCT (SEQ ID [N°] NO: 21)). The promoter *Esr2* in the form of a fragment HindIII/NotI of 2.44 kb was introduced into this adaptor. The construction thus obtained contains the gene *Esr2* in antisense orientation under the control of its own promoter (plasmid L129/46 (cf Figure 11)).

IN THE CLAIMS:

Claim 20 has been amended as follows:

20. (amended) A promoter nucleotide sequence according to claim 17, comprising a sequence chosen from amongst the sequences SEQ ID [N° 1, N° 2, N° 3, N° 4, N° 5, N° 6 and N° 7] NO: 1, NO: 2, NO: 3, NO: 4, NO: 5, NO: 6 and NO: 7, and any sequence which is a homologue of these.

SEQUENCE LISTING

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ROGOWSKY, PETER
PEREZ, PASCUAL

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ctatttttat gcattcttca aaattgacac aaattaaact aggagaattc aatacattct 180
tgccataaca tattctaatg caaatattaa gtagattgct caacatcggt acacatcttt 240
tggacgatta attagtattg tctcactaca ttctttgttt tagcagttca tcaataatgc 300
ctcaatagca tacttcattt taggaacttt atgaaaattg tcttaaaata gggccaagtc 360
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rratycantt yaaagntgay tcttmatttc ttacttccta tctttgstkg yttwngtwta 180
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<210> 21
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11/PRTS

PLANT SEED ENDOSPERM-SPECIFIC PROMOTERS

The present invention relates to controlling the expression of genes during the development of the endosperm. It concerns in particular promoter nucleotide sequences which enable expression which is both specific to the interface between the embryo and the endosperm and early during the development of the endosperm.

The endosperm, a characteristic formation of Angiosperm seeds, is a nutritive tissue for the embryo. This is a tissue which is complex in its structure and development, in particular with cereals. The central area of the endosperm consists of large cells with vacuoles, which store the reserves of starch and proteins, whilst the region surrounding the embryo is distinguished by rather small cells, occupied for the major part by cytoplasm. At the present time the function of these cells, referred to as "dense cytoplasmic cells" (Schel et al. 1984) is not known. In 1994 Opsahl et al. identified a gene expressed specifically in this small region around the maize embryo, a gene which they called *Esr* standing for "Embryo Surrounding Region".

The authors of the present invention have now isolated promoter nucleotide sequences enabling an expression of the coding sequences with which they can be bound, which is specific to the region of the endosperm surrounding the embryo in Angiosperm seeds and which intervenes particularly in the early stages of the development of the endosperm.

Such promoter sequences are particularly useful for targeting or regulating the expression of genes of interest.

In the context of an improvement to plants by transgenesis, a promoter nucleotide sequence of this type can be bound effectively to a coding sequence for a gene of interest.

The nucleotide construction, preferably inserted in a vector, can be used for transforming plant cells in a stable fashion, so that the plant thus transformed contains in its genome the gene of interest associated with the promoter sequence of the invention.

The seeds which grow, by fertilisation, from this plant also contain this transgene in their genome.

Because of its association with the promoter sequence of the invention, this transgene of interest will be expressed only in the region of the endosperm surrounding the embryo, that is to say in the dense cytoplasmic cells as mentioned above.

The expression of the transgene begins from the very first days after pollination, more precisely as from the fourth day after pollination.

The promoter sequences of invention can advantageously be selected from the group consisting of the sequences comprising the sequences SEQ ID N° 1, N° 2, N° 3, N° 4, N° 5, N° 6 or N° 7 and any nucleotide sequence which is a homologue of these.

The sequence SEQ ID N° 1 corresponds to the gene promoter *Esr1*.

The sequence SEQ ID N° 2 corresponds to the gene promoter *Esr2*.

The sequence SEQ ID N° 3 corresponds to the gene promoter *Esr3*.

The sequence SEQ ID N° 4 corresponds to the gene promoter *Esr4*.

The sequence SEQ ID N° 5 corresponds to a fragment of 499 pairs of bases on SEQ ID N° 2 (nucleotides 1995-2493).

The sequence SEQ ID N° 6 corresponds to a fragment of 507 pairs of bases on SEQ ID N° 3 (nucleotides 1202-1708).

The sequence SEQ ID N° 7 is a consensus sequence of 265 nucleotides, obtained by means of comparison between the sequences SEQ ID N° 1, N° 2 and N° 3.

"Homologous nucleotide sequence" means any nucleotide sequence which differs from the sequence SEQ ID N° 1, N° 2, N° 3, N° 4, N° 5, N° 6 or N° 7, by a substitution, deletion and/or insertion of one or more nucleotides, at positions such that these homologous nucleotide sequences preserve the property of specific promoter of the sequences SEQ ID N° 1 to N° 7.

Preferably such a homologous nucleotide sequence is identical to at least 70% of the sequences SEQ ID N° 1 to

N° 7, preferably at least 80%, preferably still at least 95%.

Homology is generally determined using a sequence analysis software (for example, the Sequence Analysis Software package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705). Similar nucleotide sequences are aligned in order to obtain the maximum degree of homology (i.e. identity). To this end, it may be necessary to artificially introduce gaps in the sequence. Once the optimum alignment has been achieved, the degree of homology (i.e. identity) is established by recording all the positions for which the nucleotides of the two compared sequences are identical, with respect to the total number of positions.

Preferentially, such homologous nucleotide sequences specifically hybridises to the sequences which are complementary to the sequences SEQ ID N° 1 to N° 7 under stringent conditions. The parameters defining the stringency conditions depend on the temperature at which 50% of the paired strands separate (Tm).

For sequences comprising more than 30 bases, Tm is defined by the equation: Tm = 81.5 + 0.41 (% G + C) + 16.6 Log (concentration in cations) - 0.63 (%formamide) - (600/number of bases) (Sambrook et al., Molecular Cloning, A Laboratory Manual, Coldspring Harbor Laboratory Press, 1989, pages 9.54-9.62).

For sequences with a length less than 30 bases, Tm is defined by the equation: Tm = 4(G + C) + 2 (A + T).

Under appropriate stringency conditions, to which the aspecific sequences do not hybridise, the hybridisation temperature is approximately 5° to 30°C, preferably 5° to 10°C below Tm, and the hybridisation buffers used are preferably solutions with a ionic strength such as a 6xSSC solution for example.

The various nucleotide sequences of the invention can be of artificial origin or not. They may be DNA sequences obtained by sieving banks of sequences by means of sensors produced on the basis of the SEQ ID N° 1 to N° 7. Such banks can be prepared by conventional techniques of molecular biology, known to persons skilled in the art.

The nucleotide sequences according to the invention can also be prepared by chemical synthesis, or by mixed methods including the chemical or enzymatic modification of sequences obtained by sieving banks.

The promoter nucleotide sequences of the invention are preferably sequences isolated from cereals, in particular maize.

The promoter nucleotide sequences according to the present invention can in particular be isolated by methods of reversed PCR or working on the genome (Devic et al., 1997).

The promoter nucleotide sequences of the invention can also comprise or be associated with a *cis* CTACACCA regulating pattern, preferably repeated in tandem, or any

other pattern comprising one or more degenerated bases having the same function.

Another object of the present invention is a nucleotide construction, referred to as an expression cassette, comprising a promoter nucleotide sequence as defined above operatively bound to at least one gene of interest.

The said gene of interest can also be associated with other regulating elements such as activators and transcription termination sequences (terminators). By way of example of a terminator which can be used in such constructions, it is possible to cite the end 3' of the gene of nopaline synthase of *Agrobacterium tumefaciens*.

The said gene of interest can for example code for a protein involved in the development of the embryo and/or of the endosperm, cell growth, the metabolism of sugars (invertase) and fatty acids and the flow of nutrients (transporters). It can also code for a toxic protein, or for a protein activating or inhibiting other genes, such as a protein inhibiting a transcription factor (repression fields of the engrailed type (Poole et al. 1985) or co-repressors for example).

According to a preferred mode, the gene of interest codes for a protein whose specific expression in the area surrounding the embryo will make it possible to act on the size of the embryo and/or its development. By way of example, this gene can code for a barnase or isopentenyl-transferase.

The gene of interest can be placed in sense or antisense orientation.

The promoter nucleotide sequence of the invention can also be associated with a marker gene, for example a gene making it possible to select a plant transformed from a plant which does not contain transfected foreign DNA. As a marker gene, it is possible to cite in particular a gene confirming resistance to an antibiotic (Herrera-Estrella et al., EMBO J. 2, 987-995 (1983)) or resistance to a herbicide (EP 242 246).

Another object of the invention is any nucleotide vector, such as a plasmid, which can be used for transforming host cells, characterised in that it comprises an expression cassette as defined above. The construction of expression vectors for the transformation is within the capability of one skilled in the art following standard techniques.

Another object of the invention is an Angiosperm plant host cell, notably a cereal, transformed by a vector according to the invention.

The invention also concerns a transgenic plant or part of a transgenic plant, in particular seed, fruit or pollen, generated from such a cell.

Amongst the cells able to be transformed according to the method of the invention, examples are cells of extensively farmed plants (maize, wheat, rape, sunflower, peas, soya, barley, etc.) or food plants and flowers. Preferentially, it is possible to choose plants known to contain large

reserves (protein, glucidic and lipidic), in particular cereal plants and oily plants.

The hybrid plants obtained by crossing plants according to the invention also form part of the invention.

Another object of the invention is a method of obtaining an Angiosperm plant having improved agronomic or nutritional qualities, comprising the steps consisting of:

- transforming at least one Angiosperm plant cell by means of a vector as defined previously;

- cultivating the cell thus transformed so as to generate a plant containing in its genome an expression cassette according to the invention.

The transformation of vegetable cells can be achieved by the techniques known to one skilled in the art.

It is possible to cite in particular the methods of direct transfer of genes such as direct micro-injection into plant embryoids (Neuhaus et coll. 1997), vacuum infiltration (Bechtold et al. 1993) or electroporation (Chupeau et coll., 1989) or direct precipitation by means of PEG (Schocher et coll., 1986) or the bombardment by gun of particles covered with the plasmidic DNA of interest (Fromm M et al., 1990).

It is also possible to infect the plant with a bacterial strain, in particular Agrobacterium. According to one embodiment of the method of the invention, the vegetable cells are transformed by a vector according to the

invention, the said cell host being able to infect the said vegetable cells by allowing the integration, in the genome of the latter, of the nucleotide sequences of interest initially contained in the above-mentioned vector genome. Advantageously, the above-mentioned cell host used is *Agrobacterium tumefaciens*, in particular according to the method described in the article by An et al., (1986), or *Agrobacterium rhizogene*, in particular according to the method described in the article by Guerche et al. (1987).

For example, the transformation of vegetable cells can be achieved by the transfer of the T region of the tumour-inducing extra-chromosome circular plasmid of *Agrobacterium tumefaciens*, using a binary system (Watson et al., 1994). To do this, two vectors are constructed. In one of these vectors the T region has been eliminated by deletion, with exception of the right and left borders, a marker gene being inserted between them to allow selection in the plant cells. The other partner of the binary system is an auxiliary plasmid Ti, a modified plasmid which no longer has any T region but still contains the virulence genes *vir* necessary to the transformation of the vegetable cell.

According to a preferred mode, it is possible to use the method described by Ishida et al. (1996) for the transformation of Monocotyledons.

According to another protocol, the transformation is achieved according to the method described by Finer et al. (1992) using the tungsten or gold particle gun.

Another object in the invention is the use of the promoter nucleotide sequences referred to previously in molecular constructions intended to improve the agronomic, food or industrial quality of a plant, by acting in particular on the size of the embryo or of the endosperm and/or its development.

This is because an early specific action on the development of the tissues of the embryo and of the endosperm can be sought: according to the relative size of one or other tissue, it would be possible to obtain seeds or fruits with a higher starch (large endosperm) and/or oil (large embryo) content, via the use respectively of stimulator genes (hormone of the cellular cycle for example) or inhibitor genes (toxic protein or transcription inhibitor for example). Endosperms without embryos could also be obtained according to this model, for industrial applications in starch making and semolina processing.

By way of example, the use of genes coding for hormones (cytokinins, auxins) of the cell cycle, under the control of the promoters described according to the invention, would make it possible to modify the processes of cellularisation and, in a correlated fashion, the development of the endosperm in the light of the work of R J Scott (1998).

Action on the accumulation of nutrients in the embryo and endosperm can also be sought, using for example, as genes of interest, genes coding for transporters of nutrients (sugar in particular), to the interfaces between mother plant/endosperm and endosperm/embryo, or genes coding for

inhibitors of these transports, for a differential accumulation of nutrients in the endosperm or embryo.

The invention therefore also relates to methods for modifying the agronomic and/nutritional qualities of a plant, through an early targeted action on the development of the embryo/endosperm, using the transformation of the plants with a vector according to the invention. In particular, it is concerned with the modification of the size and/or the development of the embryo/endosperm. It also relates to the alteration of the development of the embryo, with a view to producing seeds without embryos for cereals in particular, presenting an interest for the starch and semolina industries.

The object of the invention is more precisely the use of an expression cassette as defined previously, for obtaining a transgenic Angiosperm plant exhibiting improved agronomic or nutritional qualities.

Advantageously, the transgenic plant obtained can produce grains with starch or oil contents which are modified in comparison with a non-transformed plant.

The invention also concerns the use of the transgenic plants obtained according to the invention, or parts of these plants in particular seeds, grains and fruits for preparing derived products, in particular food products.

The products obtained, whether it be seeds with a higher oil content, flours of seeds or grains with a higher starch or oil content, also come within the scope of the invention.

Finally, the object of the invention is any composition for human or animal food prepared from the said products obtained.

The following figures and examples illustrate the invention without limiting its scope.

LEGEND TO THE FIGURES

- Figure 1 depicts a diagram illustrating the steps of the cloning of the *Esr* promoters.

- Figure 2 depicts the restriction map of the plasmid L82/34, comprising in particular the promoter *pEsr1* fused with *Gus*.

- Figure 3 depicts the restriction map of the plasmid L124.19, comprising in particular the promoter *pEsr2* fused with *Gus*.

- Figure 4 depicts the restriction map of the plasmid L127a5, comprising in particular the promoter *pEsr3* fused with *Gus*.

- Figure 5 depicts the restriction map of the plasmid L78a1, comprising in particular the promoter *pEsr1* fused with *lpt*.

- Figure 6 depicts the restriction map of the plasmid L125a2, comprising in particular the promoter *pEsr2* fused with *lpt*.

- Figure 7 depicts the restriction map of the plasmid L77a101, comprising in particular the promoter *pEsr1* fused with *Barnase*.

- Figure 8 depicts the restriction map of the plasmid L126a3, comprising in particular the promoter *pEsr1* fused with *Barnase*.

- Figure 9 shows a comparison of the sequences of the promoters of the genes *Esr1, Esr2 and Esr3* (respectively prEsr1, prEsr2 and prEsr3, or SEQ ID N° 1, SEQ ID N° 2, and SEQ ID N° 3), the preserved parts being aligned.

- Figure 10 depicts the restriction map of the plasmid pWP280.

- Figure 11 depicts the restriction maps of the plasmid L129/46, comprising notably the promoter *pEsr2* fused with the sequence *Esr2* in antisense orientation.

EXAMPLES

EXAMPLE 1:

Quantitative expression Esr1, 2, 3.

The work by Opsahl-Ferstad et al. (1997) identified by "differential display" a specific amplicon of the endosperm *Esra*1 (access number on the EMBL database: X98495) and isolated by screening complementary genome banks on hybrid line HD5*HD7 (Barloy et coll. 1989) and line A188 (Gerdes and Tracey, 1993) respectively, of the

corresponding clones. From genome sequences *Esr1g1* (access number on EMBL: X98497) and *Esr2g1* (access number on EMBL: X98499) and *Esr3g2* (access number on EMBL: X99970) in particular, 3 genes *Esr1, Esr2 and Esr3* were revealed.

The authors of the present invention assessed the relative contributions of expression of each of the genes Esr by means of RT-PCR experiments, digestion by restriction enzymes and quantification according to the methods known to persons skilled in the art, using DAP 7 and DAP 9 (day after pollination) equipment.

Quantification of the different bands identified on migration gel reveals relative contributions of 18%, 53% and 29% on average, for the transcripts of Esr1, Esr2 and Esr3 respectively. The promoter of Esr2 therefore affords the strongest quantitative expression of the gene which it controls.

EXAMPLE 2:

Isolation and cloning of the promoter sequences

As illustrated in Figure 1, fragments containing the open reading phases of Esr1, Esr2 and Esr3 (Opsahl et al., 1997) as well as upstream and downstream sequences were sub-cloned in the plasmid pBluescript SK+ (Stratagene) in accordance with the methods described in Sambrook et al. (1989). The result was the plasmid L23/7 containing a fragment SalI of 2.1 kb of λEsr1g1 and the plasmid L33/1 containing a fragment BamHl of 3.4 kb of λEsr2g1. The plasmid L33/10 containing a fragment BamHl of 1.9 kb of

λEsr2g1 and the plasmid L102c24 containing a fragment Hindlll of 4.5 kb of λE1-111. Xbal sites situated just upstream of the open reading phase (TCTAGATTCCATG) made it possible to differentiate the putative promoters of the respective open reading phases. In particular, the fragment Sall/Xbal of 0.53 kb of L23/7 was designated as a putative promoter of Esr1, the fragment Hindlll/BamHl of 2.35 kb of L33/1 (upstream part of the promoter) and the fragment BamHl/Xbal of 0.14 kb of L33/10 (downstream part of the promoter) that of the putative promoter of Esr2, the fragment Hindlll/Xbal of 1.71 kb, that of the putative promoter of Esr3 and the fragment Xbal/Xbal of 1.62 kb comprising the putative promoter of Esr4. A functional promoter Esr2 of 2.49 kb was reconstructed from the fragment Hindlll/BamHl of 2.35 kb of L33/1 and from the fragment BamHl/Xbal of 0.14 kb of L33/10, in a base plasmid of the pBSSK+ type (Stratagene). The orientation of the arrows in Figure 1 represents the orientation 5'-3'.

EXAMPLE 3:

Structure of the sequences upstream of the *Esr* genes

Comparisons between the sequences of the regions 5' showed two types of homologies: a highly preserved sequence which corresponds to a proximal sequence of 265 pairs of bases and sequences of retrotransposons in the distal part. As the sequences of retrotransposons are in different orientations and positions in the three promoters, they do not seem to fulfil a role in the expression of the Esr genes. Consequently, the 265 pairs of bases will contain all the *cis* information necessary

for an expression of specific genes of the region surrounding the embryo.

The consensus sequence (SEQ ID N° 7) was obtained after alignment of the three promoter nucleotide sequences and using Sequencher 3.1 software from Genes Codes Corporation (Ann Arbor, MI 48106).

The degenerated bases are described in the Nomenclature Committee of the International Union of Biochemistry (1985): Nomenclature for Incomplete Specified Bases in Nucleic Acid Sequences, European Journal of Biochemistry 150: 1-5.

In particular,

B = C, G or T but not A

D = A, G or T but not C

H = A, C or T but not G

K = G or T

M = A or C

N = G, A or C

R = G or A

S = C or G

V = A, C or G but not T

W = A or T

X = G, A, T or C

and Y = C or T.

A homology is also observed between the proximal regions which extend over approximately 500 pairs of bases between the promoter of *Esr2* and that of *Esr3*, as defined by the sequences SEQ ID N° 5 and N° 6.

The presence of elements acting in *cis* is sought amongst the preserved sequences, the most remarkable being CTACACCA, in tandem just 50 bases upstream of the open reading phase (Figure 9). This sequence is a good candidate for being an element responsible for a tissue-specific gene expression. The first of these repetitions is also placed in the loop of the greatest reversed repetition found in the three promoters. The sequences repeated more than twice are preserved only between the promoters *pEsr2* and *pEsr3*, in the missing region of Esr1: for example the sequences ATTCT and TTTTA, each being repeated four times, a potential transcription enhancer in the light of the lowest expression of Esr1 (Figure 9).

To demonstrate the functionality of the *cis* elements, constructs comprising deleted promoter nucleotide sequences, fused with *GUS*, were prepared.

By way of example, two techniques were used to create deletions of the promoter Esr2:

- by extensive digestion of 5′ to 3′ on the fragment Hindlll-Xbal by means of the Erase-a-base™ kit from Promega (constructions L140);

- by PCR amplification of fragments of the promoter (constructions L194).

The plasmids L190 and L194 contain deleted promoter Esr2 fused with a reporter gene Gus and a terminator in accordance with the techniques described in the following example.

For the transformation, the fragments containing the constructs, deleted promoters constructs Esr2 - Gus - ter′ were transferred into another plasmid containing the construct "promoter ubiquitin-luciferase-ter", the latter serving as an internal standard for quantifying the Gus activity and correcting the position effect of the insertion of the transgene in the genome on the expression, variable from one transformed plant to another.

The fragments of the promoter Esr2 resulting from these deletions are set out in the following table 1:

Table 1:

Chosen deletion technique	Remaining fragment of the promoter Esr2*
Digestion kit (L140)	985-2493
	1254-2493
	1865-2493
	1874-2493
	1880-2493
	2077-2493
Amplification PCR (L194)	2163-2493
	2275-2493
	2373-2493

* the numbering of the promoter Esr2 is based on the sequence pEsr2 presented in an annexe (SEQ ID N° 2) which goes from HindIII (AAGCTT or A = position 1) to XbaI (TCTAGA or A = position 2493).

To demonstrate the functionality of the promoter nucleotide sequences described above, the inventors cloned them upstream of the reporter gene GUS and used the constructs obtained for the transformation of plants.

In a preferred manner, the deleted promoters *Esr2* were obtained in accordance with the following protocols:

Firstly, deletions of 5' to 3' were effected using exonuclease III. The plasmid L124/19 containing the promoter of the gene *Esr2* coupled to the gene of the β-glucuronidase described in Example 4.1 was digested by HindIII in order to generate an initiation site for the deletions and by PstI to create a protection site against the action of the exonuclease III. The deletions were carried out with the Erase-a-base™ (Promega) kit.

Secondly, fragments of the promoter were amplified using the initiator ESRX (5'GGGGTCTAGACTGTGAAGCTATTTTCCA3' (SEQ ID N° 8)) containing the restriction site XbaI (underlined) and ESRH1 (5'GGGGAAGCTTTACATTCTTGCCATAACATA3' (SEQ ID N° 9)), ESRH2 (5'GGGGAAGCTTTTCATCAATAATGCCTCATT3' (SEQ ID N° 10)) or ESRH3 (5'GGGGAAGCTTTAATTTCTTACTTCCTATCT3' (SEQ ID N° 11)) containing the Hindlll restriction site (underlined). The amplification products digested by XbaI and HindIII replaced the entire promoter *Esr2* upstream of the gene of the β-glucuronidase in the plasmid L124/19.

The deleted promoters associated with the β-glucuronidase gene were then cloned in a plasmid containing the luciferase gene under the control of the promoter of the rice actin. The latter was obtained by cloning the fragment XhoI/NcoI of the plasmid pAct1-F4 (Mc Elroy D. et al., Mol Gen Genet., 231: 150-160, 1991) corresponding to the promoter and first intron of the rice actin, in a plasmid of the pGP214 type containing the luciferase gene and the terminator of nopaline synthase (Twell D. et al., Development 109, 705-713, 1990), digested by SalI/NcoI. An adaptor containing the restriction sites SalI and NotI, formed in nucleotides 5'GGCCAGTCGACAAAGCGGCCGCATGCA3' (SEQ ID N° 12) and 5'TCAGCTGTTTCGCCGGCGT3' (SEQ ID N° 13) was introduced into the plasmid obtained, digested by NotI and PstI (plasmid L210). The fragments SalI/NotI containing the deleted promoters associated with the β-glucuronidase gene were cloned in the plasmid L210 digested by SalI and NotI.

EXAMPLE 4:

Preparation of chimeric constructs

All the constructions can be effected in particular according to the methods described in Sambrook et al. (1989). The adaptors which can be used by way of example for cloning these fragments upstream of the different effecting genes are described in the restriction maps of the corresponding plasmids.

4-1 GUS chimeric constructs

The plasmid L23/7 (Esr1) was deleted from a fragment SacI containing undesirable restriction sites. Then a fragment XbaI/EcoRI of 2164 pairs of bases of the plasmid pBl101 (Jefferson et al., 1987) containing a *Gus* gene (coding for the β-glucuronidase but with no promoter) and a terminating sequence *nos*, was introduced. The new plasmid thus formed was then digested by XhoI and the digestion product containing the promoter region associated with the *Gus* gene and positioned upstream of the latter was subcloned in the vector pBCKS+ (Stratagene) so that the promoter is close the hybridisation zone of the initiator T7, thus enabling the plasmid L82/34 to be obtained (Figure 2, Table 2).

According to a similar protocol and with the restriction enzymes indicated in the corresponding figures, it was possible to obtain the plasmids L124/19 (pEsr2-GUS, Figure 3, Table 3) and L127a5 (pEsr3-GUS, Figure 4, Table 4).

It is also possible to use other reporter genes in replacement for GUS, for example GFP (Green Fluorescent

Protein, Siemering KR et al., 1996), to confirm the results obtained with GUS.

According to a protocol similar to that described previously, the fragment HindIII-XbaI of the promoter pEsr2 was fused with the coding sequence for GFP.

Table 2: Characteristics of the plasmid L82/34

Fragment	Position	Reference
pEsr1	741-1272	
Gus	1302-3107	
ter nos	3181-3434	
cat	5878-5223	
pBCKS+	1-740	Stratagene
L23/7 (pEsr1)	741-1272	Opsahl-Ferstad et al., 1997* and this example
pBI101	1273-3436	Jefferson et al., 1987
L23/7	3437-3763	Opsahl-Ferstad et al., 1997 and this example
(upstream)	3764-3769	Stratagene
pBSSK+	3770-6428	Stratagene
pBCKS+		

* the insert corresponds to the fragment Esr1g1 drawn in Figure 4 of Opsahl-Ferstad et al., 1997

Table 3: Characteristics of the plasmid L124/19

Fragment	Position	Reference
pEsr2	689-3175	
Gus	3205-5010	
ter nos	5084-5337	
bla	7441-6584	
pBSSK+	1-674	Stratagene
Linker JFB 34	675-688	this example [1)]
L33/1 (pEsr2')	689-3037	this example**
L33/10(pEsr2")	3038-3175	Opsahl-Ferstad et al., 1997 and this example**
pBI101	3176-5339	Jefferson et al., 1987
linker JFB56	5340-5346	this example [2)]
pBSSK+	5347-7566	Stratagene

** the inserts are presented partly as a fragment Esr2g1 in Figure 4 of Opsahl-Ferstad et al., 1997

[1)] adaptor JFB34: 5'TCGACTGCAGCCCA 3' (SEQ ID N° 14)
3'GACGTCGGGTTCGA 5' (SEQ ID N° 15)

[2)] adaptor JFB56: 5'CTAGACCCGAATTCGC 3' (SEQ ID N° 16)
3'TGGGCTTAAGCGCCGG 5' (SEQ ID N° 17)

Table 4: Characteristics of the plasmid L127a5

Fragment	Position	Reference
pEsr3	689-2390	
Gus	2420-4225	
ter nos	4229-4552	
bla	6656-5799	
Pbssk+	1-674	Stratagene
Linker JFB 34	675-688	this example
L102c24 (pEsr3)	689-2390	this example
pBI101	2391-4554	Jefferson et al., 1987
linker JFB56	4555-4561	this example
pBSSK+	4562-6781	Stratagene

4-2 Chimeric constructs lpt

The lpt gene codes for isopentenyl-transferase, which is an enzyme involved in the synthesis of cytokinine, a phytohormone implicated in vegetable cell growth. The gene sequence was determined by Heidekamp F. et al. (1983). Prior works also showed that this sequence, under the control of a specific promoter of the ovule, made it possible to increase the dry matter content in the fruit, in tomatoes, Martineau B. et al. (1995).

According to the cloning methods described above and with the fragments of nucleic acids and restriction enzymes indicated in the corresponding figures, it was possible to prepare constructs pEsr1-lpt (Figure 5, Table 5) and pEsr2-lpt (Figure 6, Table 6). It is also possible to obtain a construct pEsr3-lpt, according to the same protocol. For preparing these constructs, NcoI (CCATGG) sites straddling the codon ATG of the start of the open reading phase were used instead of the XbaI sites.

Table 5: Characteristics of the plasmid L78a1

Fragment	Position	Reference
pEsr1	310-844	
ipt	846-1565	
ter ipt	1566-	
bla	1850	
	2963-	
	3823	
pUC118	1-231	Boehringer
L23/7 (pEsr1)	232-844	Opsahl-Ferstad et al., 1997 and this example
isolated mutation	845	Zhang et al., 1996
pRZ1	846-1883	Zhang et al., 1995
pUC118	1884-	Boehringer
	4763	

Table 6: Characteristics of the plasmid L125a2

Fragment	Position	Reference
pEsr2	689-3183	
ipt	3185-3904	
ter ipt	3905-4189	
bla	6309-5449	
pBSSK+	1-674	Stratagene
Linker JFB 34	675-688	this example
L33/1 (pEsr2')	689-3037	this example
L33/10 (pEsr2")	3038-3183	Opsahl-Ferstad et al., 1997 and this example
isolated mutation	3184	Zhang et al., 1996
pRZ1	3185-4198	Zhang et al., 1995
adaptor JFB56	4199-4214	this example
pBSSK+	4215-6434	Stratagene

4-3 Barnase chimeric constructs

The barnase gene codes for an Rnase. This gene was isolated using Bacillus amyloliquefaciens (Hartley, 1988). Its use for creating sterile male plants was described in

the application EP 344 029 published by Mariani et al. (1990).

In the context of the invention, the plasmids L77a101 (pEsr1-barnase) and L126a3 (pEsr2-barnase) described in Figures 7 (Table 7) and 8 (Table 8) were obtained from the plasmid "promoter A6-barnase" described in WO 92/11379, by replacing pA6 with the promoters pEsr1 and pEsr2 respectively, in accordance with the techniques known to persons skilled in the art.

It is also possible to obtain a construct pEsr3-Barnase, in accordance with a similar protocol.

Table 7: Characteristics of the plasmid L77a101

Fragment	Position	Reference
pEsr1	80-605	
Barnase	613-945	
ter CaMV	1571-2257	
bla	4324-3467	
pA3	1-28	Scott et al., 1992
L23/7 (pEsr1)	29-605	Opsahl-Ferstad et al., 1997 and this example
pA3	606-4473	Scott et al., 1992

Table 8: Characteristics of the plasmid L126a3

Fragment	Position	Reference
pEsr2	43-2529	
Barnase	2537-2869	
ter CaMV	3495-4181	
bla	6248-5391	
pA3	1-28	Scott et al., 1992
linker JFB34	29-42	this example
L33/1 (pEsr2')	43-2391	this example
L33/10	2392-2529	Opsahl-Ferstad et al., 1997 and this example
(pEsr2")	2530-6397	Scott et al., 1992
pA3		

4-4 Chimeric construct antiEsr2

The reconstituted functional promoter Esr2 (2.49 kb), described in Example 2 and chosen preferentially in the light of the quantitative expression results described in Example 1, was fused with the sequence Esr2g2 (Opsahl et al., 1997) taken in antisense orientation, itself fused with the terminator Nos.

In a preferred manner, the chimeric construct containing the gene *Esr2* in the reverse direction under the control of its own promoter was obtained in accordance with the following protocol:

In a plasmid derived from pJIT30 containing the promoter *35S*, a multiple cloning site and the terminating sequence of the cabbage mosaic virus (Guerineau F. et al., Plant Mol Biol, 15: 127-136, 1990), an adaptor containing a SpeI site and formed by the oligonucleotides (5'GATCCACTAGTCCCG (SEQ ID N° 18)) and (5'AATTCGGGACTAGTG (SEQ ID N° 19)) was

inserted between the sites BamHI and EcoRI. The fragment EcoRI/SpeI of the plasmid L42 a14 (Opsahl-Ferstad et coll., 1997) was inserted in the plasmid described previously. The construction thus obtained contained the gene *Esr2* in antisense orientation under the control of the promoter 35S (plasmid L79 b5).

The promoter *35S* was eliminated in the plasmid L79 b5 by restriction by SacI and HindIII, and replaced by an adaptor containing the restriction site HindIII and NotI and formed by the oligonucleotides (5'AAGCTTTTTGCGGCCGC (SEQ ID N° 20)) and (5'TCGAGCGGCCGCAAAAAGCTTAGCT (SEQ ID N° 21)). The promoter *Esr2* in the form of a fragment HindIII/NotI of 2.44 kb was introduced into this adaptor. The construction thus obtained contains the gene *Esr2* in antisense orientation under the control of its own promoter (plasmid L129/46 (cf Figure 11)).

According to a similar protocol, it is possible to obtain the constructs comprising the promoter Esr2 fused with the antisense sequences Esr1 and Esr3 respectively. It is also possible to obtain the same type of chimeric constructs with the other Esr promoters according to the invention.

Constructs comprising the constituent promoter 35S fused with the Esr antisense sequences described below have also been obtained.

EXAMPLE 5:

Obtaining transgenic plants (necessity for the stable transformation of maize)

Transient expression experiments using transformation by bombardment of vegetable cells, with chimeric constructs pEsr-GUS and constituent promoter-GUS respectively, did not give results revealing the specificity of expression of the promoters tested: no GUS activity was displayed in the area defined by the Esr cells. The small size of this area and other peculiar particularities could explain the fact that the technique is unsuited under standard conditions to transient expression. By way of example, the constituent promoters tested as a control are the rice actin promoters (McElroy et al., 1992), maize ubiquitin (Christensen et al., 1996), maize Adh (Dennis et al., 1984) and 35S (Odell et al., 1985), gave a blue colouring throughout the endosperm, demonstrating the functionality of the transformation system, but not in the area surrounding the embryo, which confirms the unsuitability of the system for this area.

The transformation aimed at a stable expression therefore became necessary for studying the specificity of expression of the promoters according to the invention.

5-1 Particle gun

The method used is based on the use of a particle gun identical to the one described by J. Finer (1992). The target cells are undifferentiated cells in rapid divisions which have preserved suitability for the regeneration of entire plants. This type of cell composes the embryogenic callus (referred to as type II) of maize. These calluses are obtained from immature embryos of the genotype Hill according to the method and on the media described by

Armstrong (Maize Handbook: 1994, M. Freeling, V. Walbot Eds, pp. 665-671). These fragments of the calluses with a surface area of 10 to 20 mm^2 were disposed, 4 hours before bombardment, at the rate of 16 fragments per dish, in the centre of a Petri dish containing a culture medium identical to the initiation medium, with 0.2 M of mannitol + 0.2 M of sorbitol added. The plasmids described in the previous examples and carrying the genes to be introduced are purified on a Qiagen^R column following the instructions of the manufacturer. They are then precipitated on particles of tungsten (M10) in accordance with the protocol described by Klein (1987). The particles thus coated are projected towards the target cells by means of the gun and in accordance with the protocol described by J. Finer (1992). The dishes of calluses thus bombarded are then sealed by means of Scellofrais^R and then cultivated in darkness at 27°C. The first planting out took place 24 hours afterwards, and then every fortnight for 3 months on a medium identical to the initiation medium with a selective agent added. After 3 months or sometimes earlier, calluses are obtained whose growth is not inhibited by the selective agent, normally and for the major part composed of cells resulting from the division of a cell which integrated in its genotype one or more copies of the selection gene. The frequency of obtaining such calluses is approximately 0.8 callus per dish bombarded.

These calluses are identified, individualised, amplified and then cultivated so as to regenerate plant germs, modifying the hormonal and osmotic balance of the cells in accordance with the method described by Vain et al. (1989). These plants are then acclimatised in a

greenhouse, where they can be crossed in order to obtain hybrids or self-fertilised.

5-2 Transformation by *Agrobacterium*

Another transformation technique which can be used in the context of the invention uses *Agrobacterium tumefaciens*, in accordance with the protocol described by Ishida et al. (1996), in particular from immature embryos from 10 days after fertilisation. All the media used are referenced in the reference cited. The transformation begins with a co-culture phase in which the immature embryos of the maize plants are brought into contact for at least 5 minutes with *Agrobacterium tumefaciens* LBA 4404 containing the superbinary vectors. The superbinary plasmid is the result of a homologous recombination between an intermediate vector carrying ADN-T containing the gene of interest and/or the selection marker derived from the plasmids described in the previous examples, and the vector pSB1 of Japan Tobacco (EP 672 752) which contains: the genes virB and virG of the plasmid pTiBo542 present in the supervirulent strain A281 of *Agrobacterium tumefaciens* (ATCC 37349) and a homologous region found in the intermediate vector allowing this homologous recombination. The embryos are then placed on a medium LSAs for 3 days in darkness and at 25°C. A first selection is effected on the transformed calluses. The embroygenic calluses are transferred onto a medium LSD5 containing phosphinotricine at 5 mg/l and cefotaxime at 250 mg/l (elimination or limitation of the contamination by *Agrobacterium tumefaciens*). This step is carried out 2 weeks in darkness and at 25°C. The second selection step is carried out by the transfer of the embryos which are

developed on an LSD5 medium, on an LSD10 medium (phosphinotricine at 10 mg/l) in the presence of cefotaxime, for 3 weeks under the same conditions as before. The third selection step consists of excising the type I calluses (fragments of 1 to 2 mm) and transferring them 3 weeks in darkness and at 25°C onto an LSD 10 medium in the presence of cefotaxime.

The regeneration of the plant germs is carried out by excising the type I calluses which have proliferated and transferring them onto an LSZ medium in the presence of phosphinotricine at 5 mg/l and cefotaxime for 2 weeks at 22°C and under continuous light.

The plant germs which have regenerated are transferred onto an RM + G2 medium containing 100 mg/l of Augmentin for 2 weeks at 22°C and under continuous illumination for the development step. The plants obtained are then transferred to the phytotron with a view to their acclimatisation.

5-3 Preferred mode for the barnase constructs: retransformation of the act-barstar calluses

The barnase chimeric constructs described in Example 3 can be used for conventional transformations according to one or other of the techniques described above.

According to a preferred mode, adapted to the toxic character of barnase, pretransformed calluses are used for the transformation, containing the gene barstar, which codes for a specific inhibitor of Barnase (Hartley, 1988). This gene serves as "protection" during the process of

regenerating these calluses, which takes place essentially from embryogenesis in maize.

- step a: obtaining a line expressing barstar and a plasmid containing the gene for resistance to hygromycine: A first transformation step is carried out in accordance with one of the protocols described, with the plasmid pWP280 containing the cassette pActin-intron-Barstar-Nos poly A.

This cassette was obtained according to the following steps: the barnase fragment was amplified with PCR from the plasmid pTG2 (Horovitz et al., 1990) and then subcloned as a fragment XbaI/HindIII in the plasmid pBluescript KS+ (Stratagene) giving the plasmid pWP118.

The barstar gene was then transferred as a fragment XbaI/HincII into a site XbaI/SmaI of the plasmid pW90, derived from the plasmid pJlT30 described by Guerineau et al. (1990) (promoter 35SCaMV replaced by the double promoter 35S and the *polylinker* region between the sites XbaI and EcoRI replaced by the sites SpeI, BamHI, SmaI and PstI).

The region polyA CaMV of the plasmid obtained is replaced by the region nos polyA of pED23 (Dale et al., 1991) forming the plasmid pWP266. Finally, the double promoter region 35S CaMV is replaced by the rice actin promoter and the intron derived from pCOR113 (Mc Elroy et al., 1991) forming the plasmid pWP280 (Figure 10).

The "actin-barstar promoter" plants thus produced are analysed by Northern Blot in order to identify the plants

correctly expressing ARNm coding for Barstar. The plants thus produced supply embryos expressing the Barstar gene, which will be used for producing type II calluses according to known techniques: putting the embryos in culture on a medium inducing callogenesis and replanting on a selective medium containing hygromycin.

- step b: transformation of these calluses with the barnase chimeric construct:

The act-barstar calluses obtained at the previous step are then bombarded according to the technique described at point 5-1 with the "Esr-barnase promoter" construct previously described with a plasmid conferring resistance to Basta (pDM302, Mc Elroy et al., 1991). The two genes are then separated into the descendants by segregation, in order to see the effect of the single promoter construct Esr-barnase.

Better results, particularly with regard to the effectiveness of transformation and the number of plants regenerated, were obtained according to this preferred mode, in comparison with the conventional technique which aims to transform the calluses directly by means of the "Esr-barnase" constructs.

EXAMPLE 6

Demonstration of the functionality of the promoter sequences (the case of GUS constructs)

In order to detect the β-glucuronidase activity, the maize seeds issuing from plants transformed by the particle gun

are harvested at precise stages of the development and cut along the longitudinal axis. They are incubated in the presence of 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-GlcA, Duchefa), at 37°C for 24 hours (Jefferson et al., 1987).

In the case of the construct Esr2-Gus in particular, the blue colouring is delimited at the contour of the embryo at the 4^{th} and 5^{th} days after pollination, and then only at the suspensor level on the 6^{th} and 7^{th} days, and finally at the base of the suspensor at days 9, 12, 13 and 15.

The expression results in the transgenic plants demonstrate that the fragments 5' described in the present invention correspond to functional promoters and that they are sufficient for a correct spatio-temporal expression, in accordance with the prior results of Opsahl et al. (1997).

The use of these promoter nucleotide sequences in molecular constructions intended to improve the agronomic, food or industrial quality of a plant is particularly advantageous for modifying the size of the embryo or of the endosperm and/or its development.

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CLAIMS

1. An isolated promoter nucleotide sequence allowing an expression of the coding sequences to which it is bound, said expression being i) specific to the region of the endosperm surrounding the embryo in the seeds of the angiosperms and ii) intervening in particular in the early stages of development of the endosperm.

2. The promoter nucleotide sequence according to Claim 1, isolated from cereals, in particular maize.

3. The promoter nucleotide sequence according to either one of Claims 1 or 2, comprising a sequence selected from the group consisting of sequence SEQ ID N° 1, N° 2, N° 3, N° 4, N° 5, N° 6 or N° 7, and any sequence which is a homologue of these.

4. The promoter nucleotide sequence according to any one of the preceding claims, also comprising a regulator element in *cis* defined by the pattern CTACACCA, preferably repeated in tandem.

5. An expression cassette comprising a promoter nucleotide sequence according to any one of the preceding claims, operatively bound to at least one gene of interest.

6. Expression cassette according to Claim 5, in which the gene of interest codes for a protein selected from the group consisting of a protein involved in the development of the embryo and/or of the endosperm, the cell growth,

the metabolism of the sugars or that of the fatty acids, a toxic protein, and a transcription inhibiting protein.

7. An expression cassette according to either one of Claims 5 or 6, in which the gene of interest codes for a first protein chosen from amongst barnase or isopentenyltransferase.

8. An expression vector containing an expression cassette according to any one of Claims 5 to 7.

9. An angiosperm plant host cell, particularly cereal, transformed by a vector according to Claim 8.

10. A transgenic plant or part of a transgenic plant, in particular fruit, seed, grain or pollen, generated from a cell according to Claim 9.

11. The plant or part of a plant according to Claim 10, wherein said plant or part of plant is a cereal or oily plant, selected in particular from the group consisting of maize, wheat, rape and sunflower, preferentially maize.

12. A hybrid transgenic plant obtained by crossing plants as defined in either one of Claims 10 or 11.

13. A method of obtaining an angiosperm plant having improved agronomic or nutritional qualities, comprising the steps consisting of:

- transforming at least one angiosperm plant cell by means of a vector according to Claim 8;

- cultivating the cell thus transformed so as to generate a plant containing in its genome an expression cassette according to any one of Claims 5 to 7.

14. Use of an expression cassette as defined in any one of Claims 5 to 7, for obtaining a transgenic angiosperm plant exhibiting improved agronomic or nutritional qualities.

15. The use according to Claim 14, for obtaining a transgenic plant producing seeds with starch or oil contents modified compared with a non-transformed plant.

16. Use of a plant or part of a plant, in particular a seed, grain or fruit, as defined in any one of Claims 10 to 12, for the preparation of derived products, notably food products.

Fig. 1

Genome bank HD5 x HD7 (Esra sensor)

5

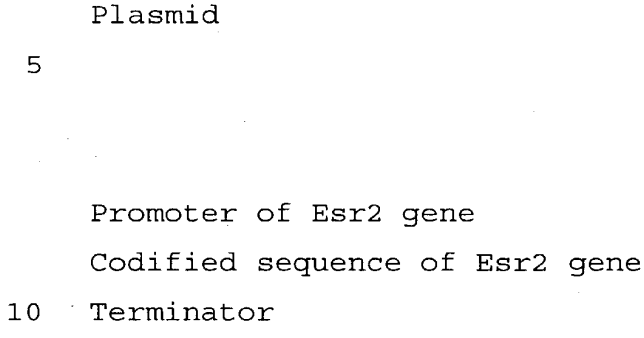
Fig. 9

Majority

Repetition CTACACC in tandem

Repetition TTTTA → Repetition ATTCT

Fig. 11


Plasmid
Promoter of Esr2 gene
Codified sequence of Esr2 gene
Terminator

LIST OF SEQUENCES

5 Specific promoters of the endosperm of vegetable seeds

Pages 4-7

Artificial sequence

Description of the artificial sequence: oligonucleotide

etc.

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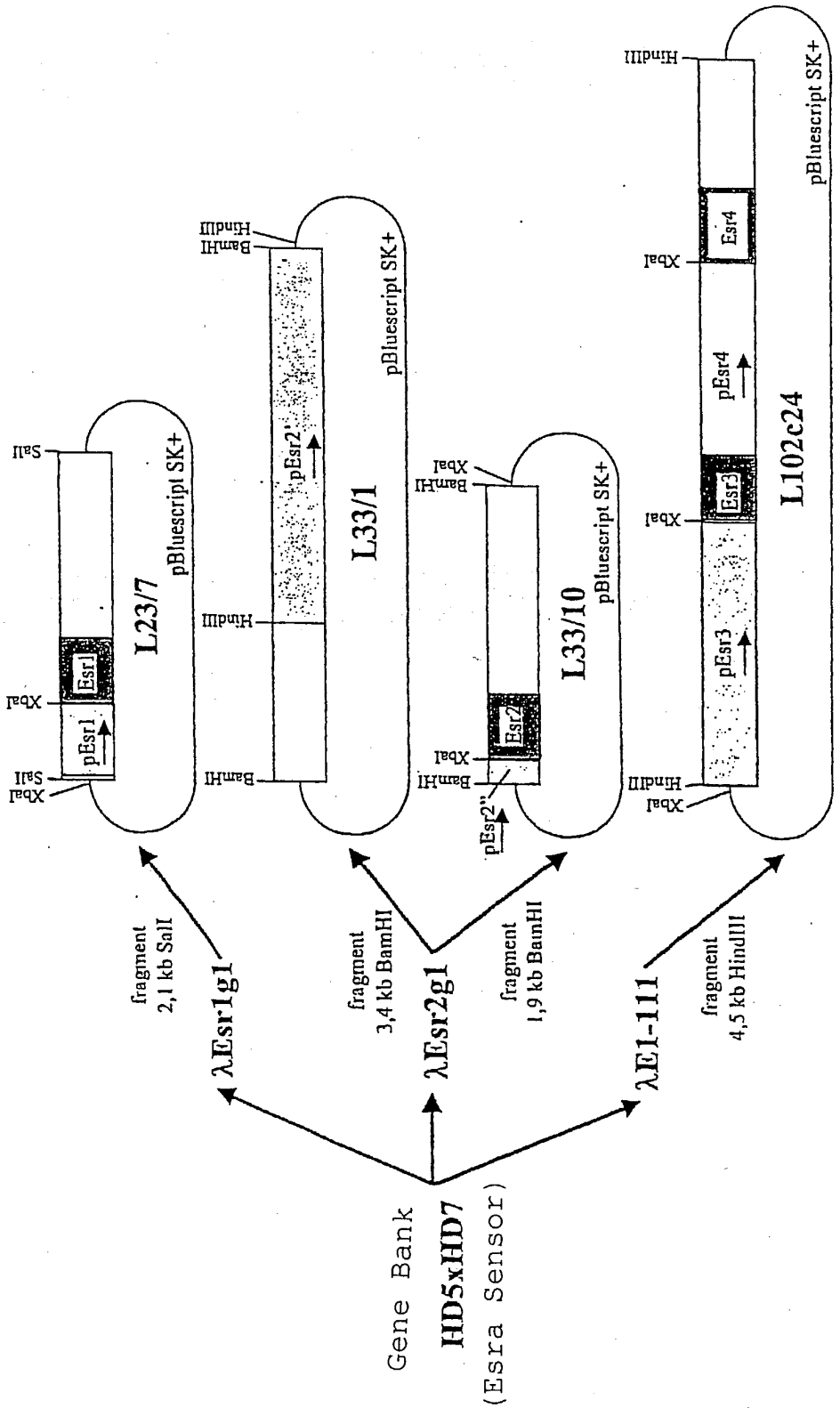
(54) **Title:** PLANT SEED ENDOSPERM-SPECIFIC PROMOTER

(54) **Titre:** PROMOTEURS SPECIFIQUES DE L'ALBUMEN DES GRAINES DE VEGETAUX

(57) **Abstract:** The invention concerns promoter nucleotide sequences enabling expression of encoding sequences whereto they can be bound, which is specific of the endosperm region enclosing the embryo of seeds of Angiosperms and which intervene in particular in the early stages of endosperm development, and their use for agronomic or nutritional improvement of plants.

(57) **Abrégé:** Cette invention concerne des séquences nucléotidiques promotrices permettant une expression des séquences codantes auxquelles elles peuvent être liées, qui est spécifique de la région de l'albumen entourant l'embryon dans les graines des Angiospermes et qui intervient en particulier dans les stades précoces du développement de l'albumen, ainsi que leur utilisation pour l'amélioration agronomique ou nutritionnelle des plantes.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(Esra Sensor)
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λEsr2g1
λE1-111
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fragment 3,4 kb BamHI
fragment 1,9 kb BamHI
fragment 4,5 kb HindIII
L23/7
pBluescript SK+
SalI
XbaI
Esr1
pEsr1
L33/1
pBluescript SK+
BamHI
HindIII
pEsr2'
L33/10
pBluescript SK+
BamHI
XbaI
Esr2
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HindIII
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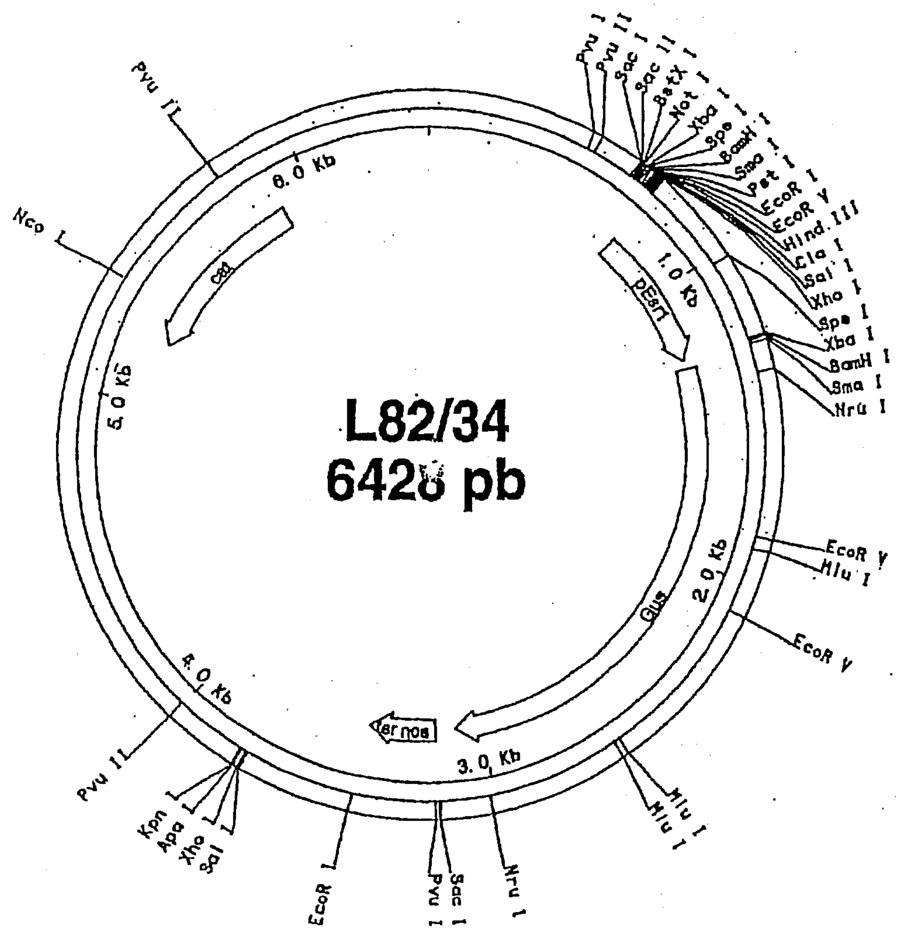
Esr4
pEsr4
Esr3
pEsr3

FIG.1



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664	Sac II
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697	Sma I
705	Pst I
707	EcoR I
715	EcoR V
719	Hind III
726	Cla I
734	Sal I
740	Xho I
1024	Spe I
1272	Xba I
1278	BamH I
1285	Sma I
1375	Nru I
1859	EcoR V
1897	Mlu I
2090	EcoR V
2592	Mlu I
2610	Mlu I
3016	Nru I
3169	Sac I
3183	Pvu I
3436	EcoR I
3763	Sal I
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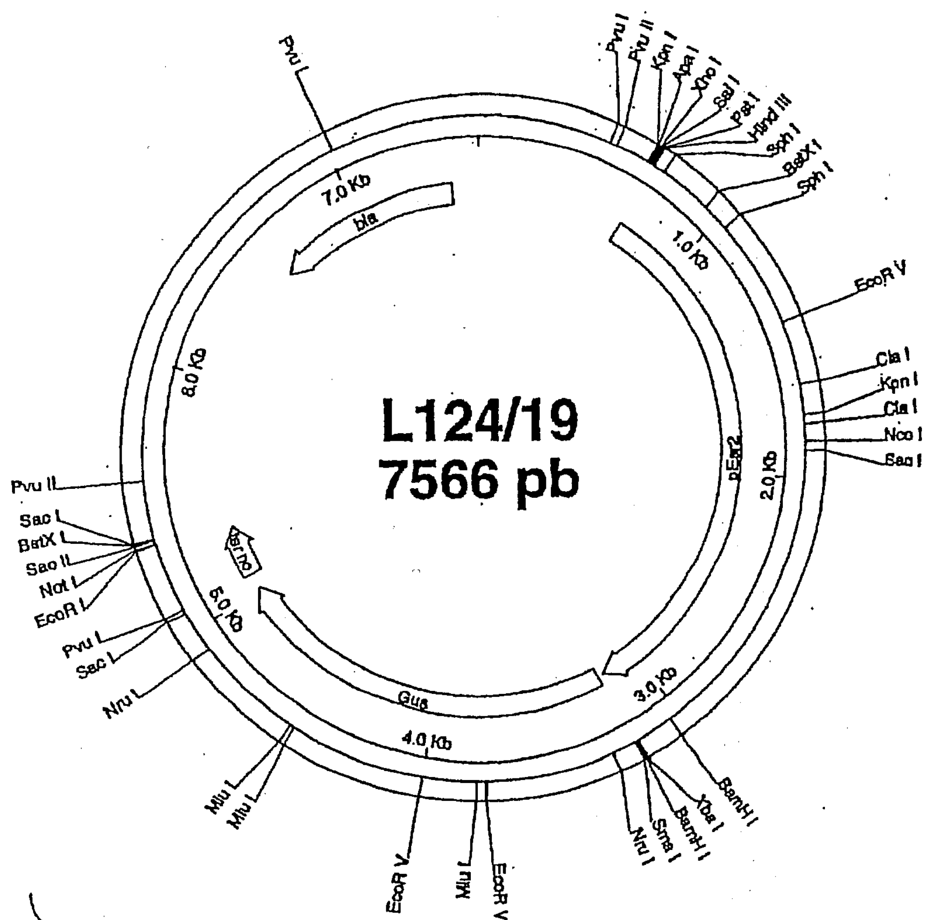
6764	Pvu II

FIG.2



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668	Xho I
674	Sal I
683	Pst I
688	Hind III
730	Sph I
925	BstX I
1033	Sph I
1443	EcoR V
1675	Cla I
1779	Kpn I
1806	Cla I
1870	Nco I
1901	Sac I
3037	BamH I
3176	Xba I
3181	BamH I
3188	Sma I
3278	Nru I
3762	EcoR V
3800	Mlu I
3993	EcoR V
4495	Mlu I
4513	Mlu I
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7024	Pvu I

FIG.3

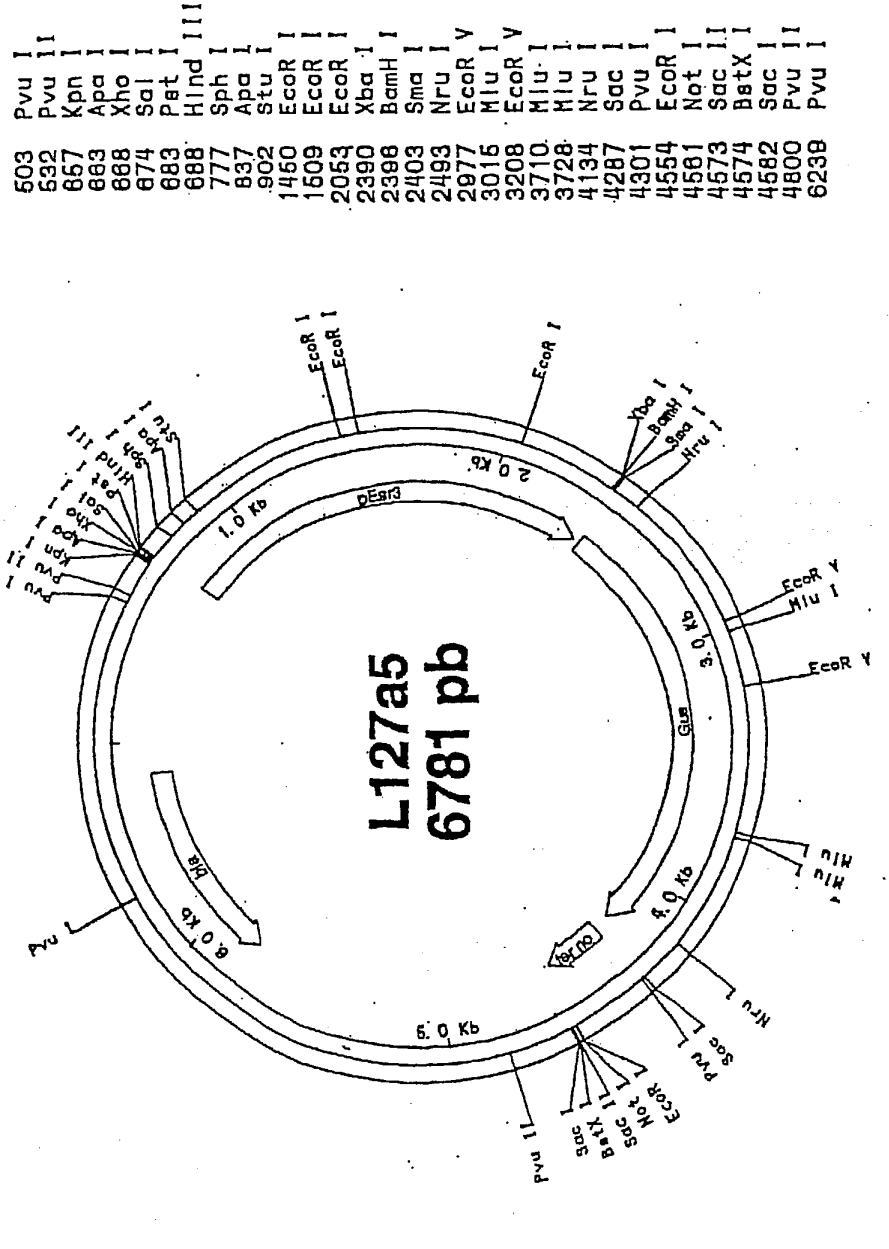
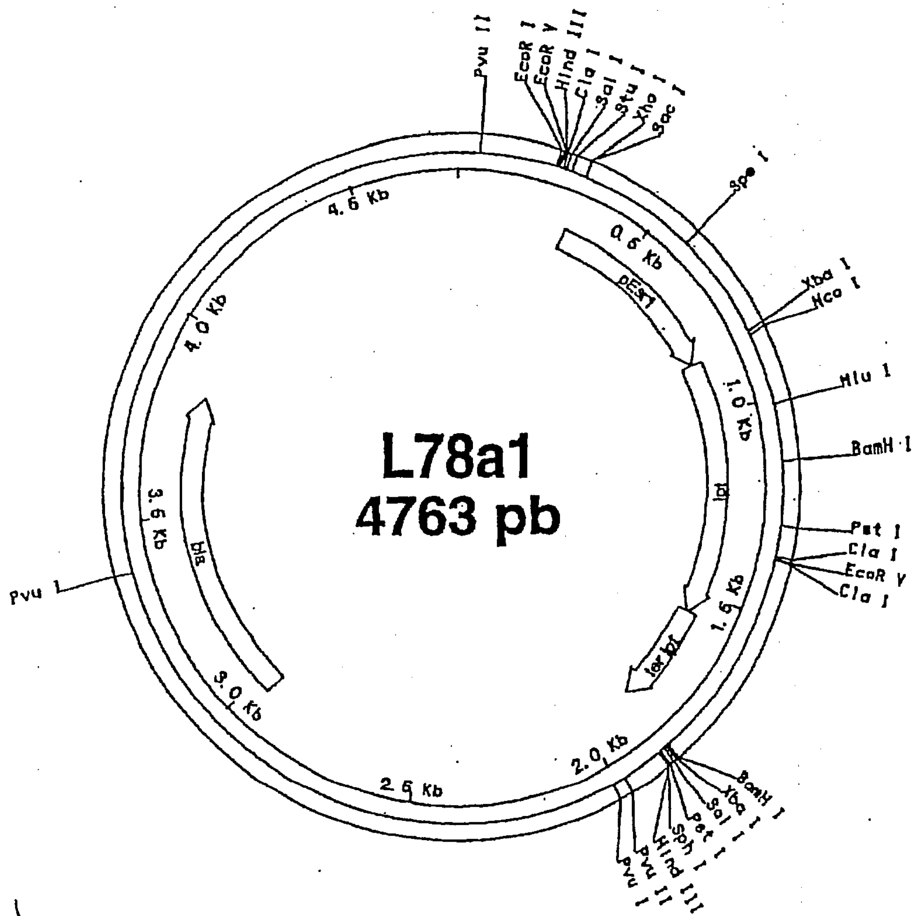


FIG.4



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243	Hind.III
250	Cla I
258	Sal I
269	Stu I
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305	Sac I
588	Spe I
836	Xba I
844	Nco I
1014	Mlu I
1137	BamH I
1286	Pst I
1357	Cla I
1365	EcoR V
1367	Cla I
1853	BamH I
1859	Xba I
1865	Sal I
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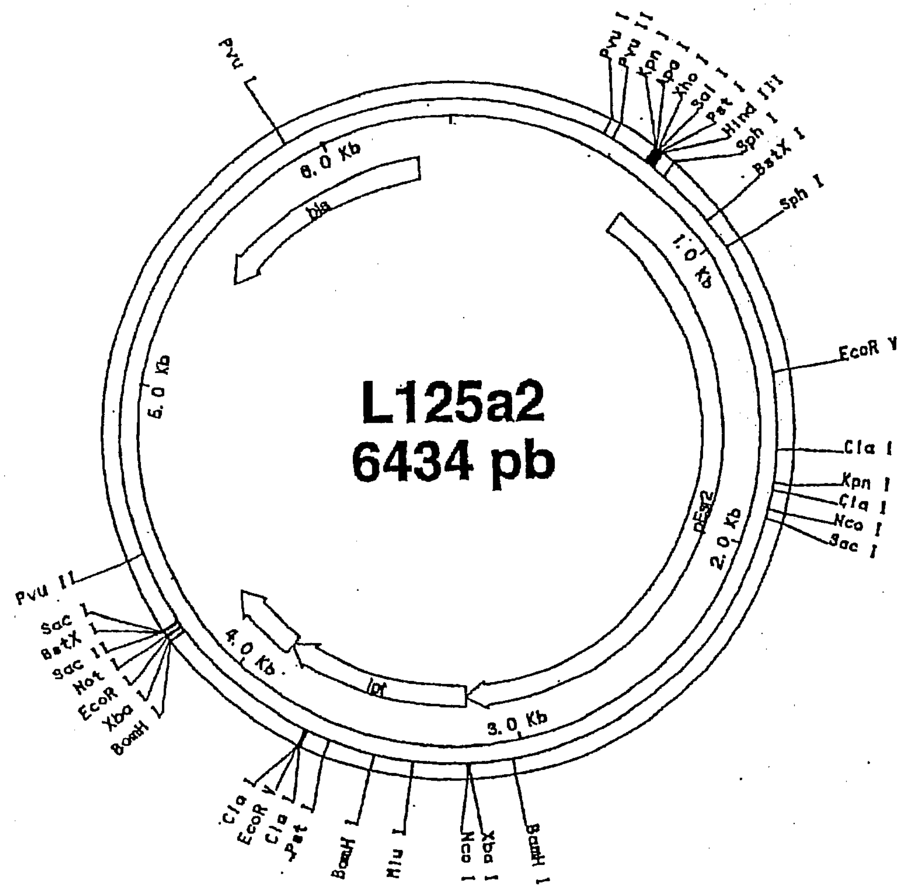
1883	Hind III
1978	Pvu II
2009	Pvu I
3381	Pvu I

FIG.5



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1901	Sac I
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3183	Nco I
3353	Mlu I
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3625	Pst I
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3706	Cla I
4192	BamH I
4198	Xba I
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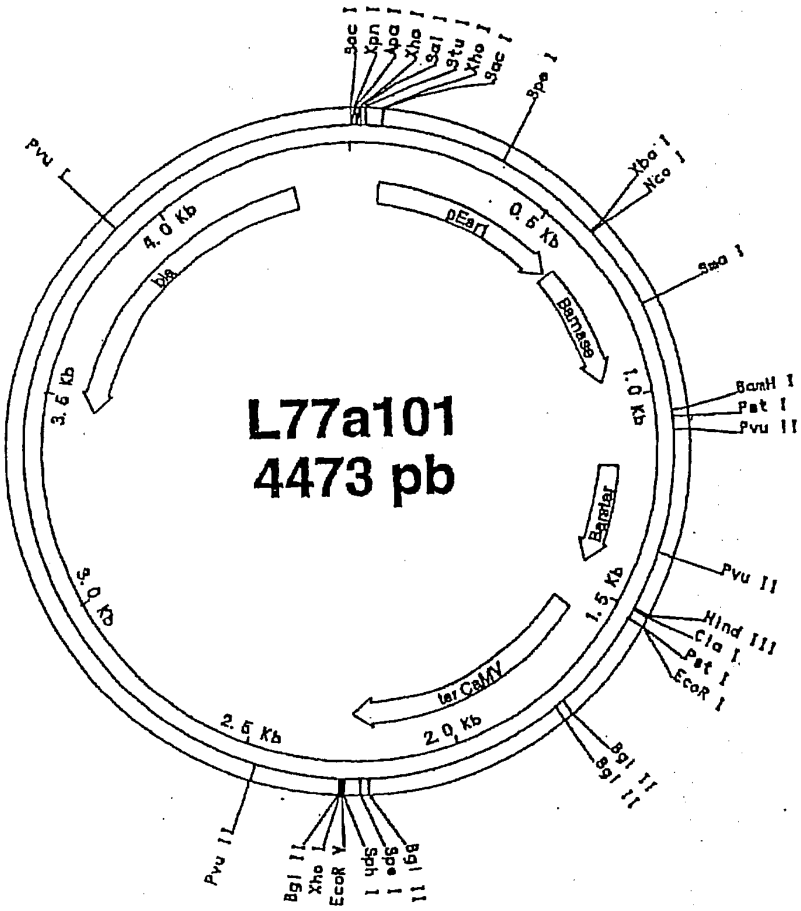
4453	Pvu II
5892	Pvu I

FIG.6



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28	Sal I
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75	Sac I
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611	Nco I
806	Sma I
1047	BamH I
1065	Pst I
1094	Pvu II
1362	Pvu II
1492	Hind III
1499	Cla I
1517	Pst I
1519	EcoR I
1745	Bgl II
1765	Bgl II
2217	Bgl II
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2273	EcoR V
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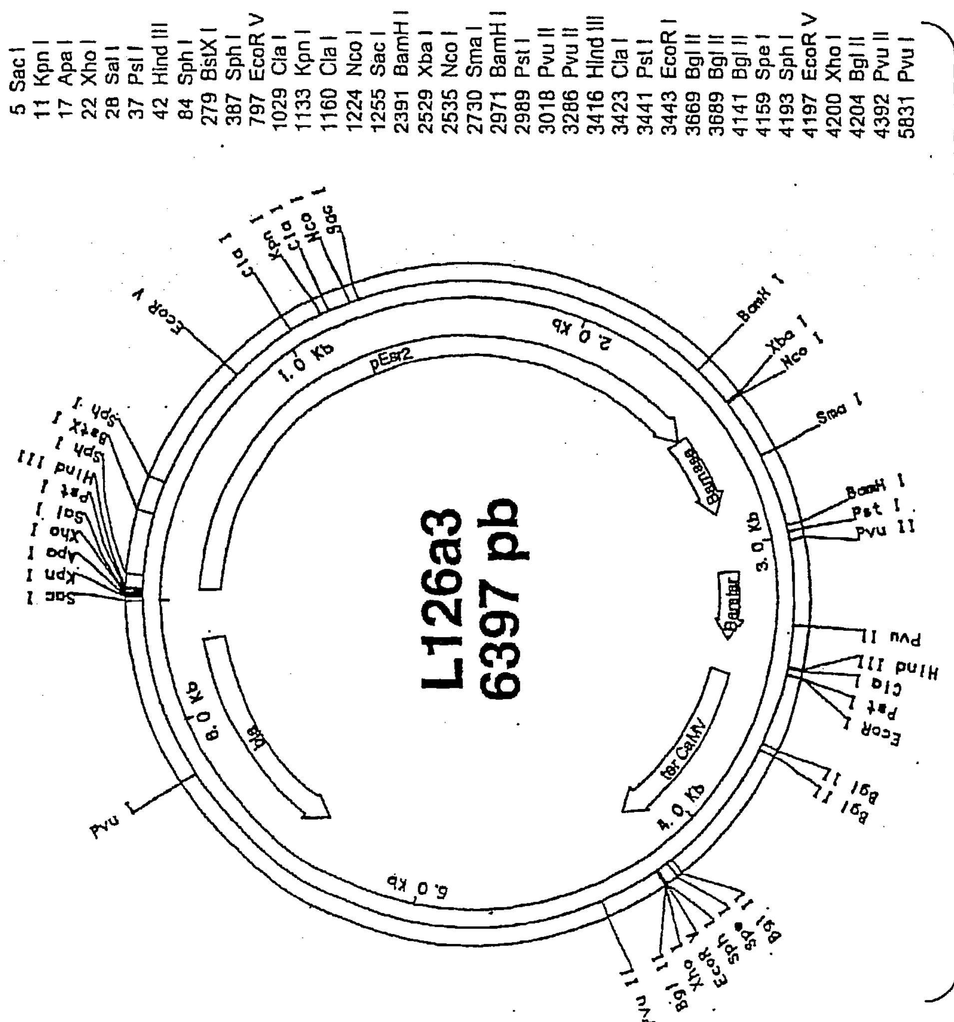
2280	Bgl II
2468	Pvu II
3907	Pvu I

FIG.7



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17 Apa I
22 Xho I
28 Sal I
37 Pst I
42 Hind III
84 Sph I
279 BstX I
387 Sph I
797 EcoR V
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1160 Cla I
1224 Nco I
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2535 Nco I
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2989 Pst I
3018 Pvu II
3286 Pvu II
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FIG.8

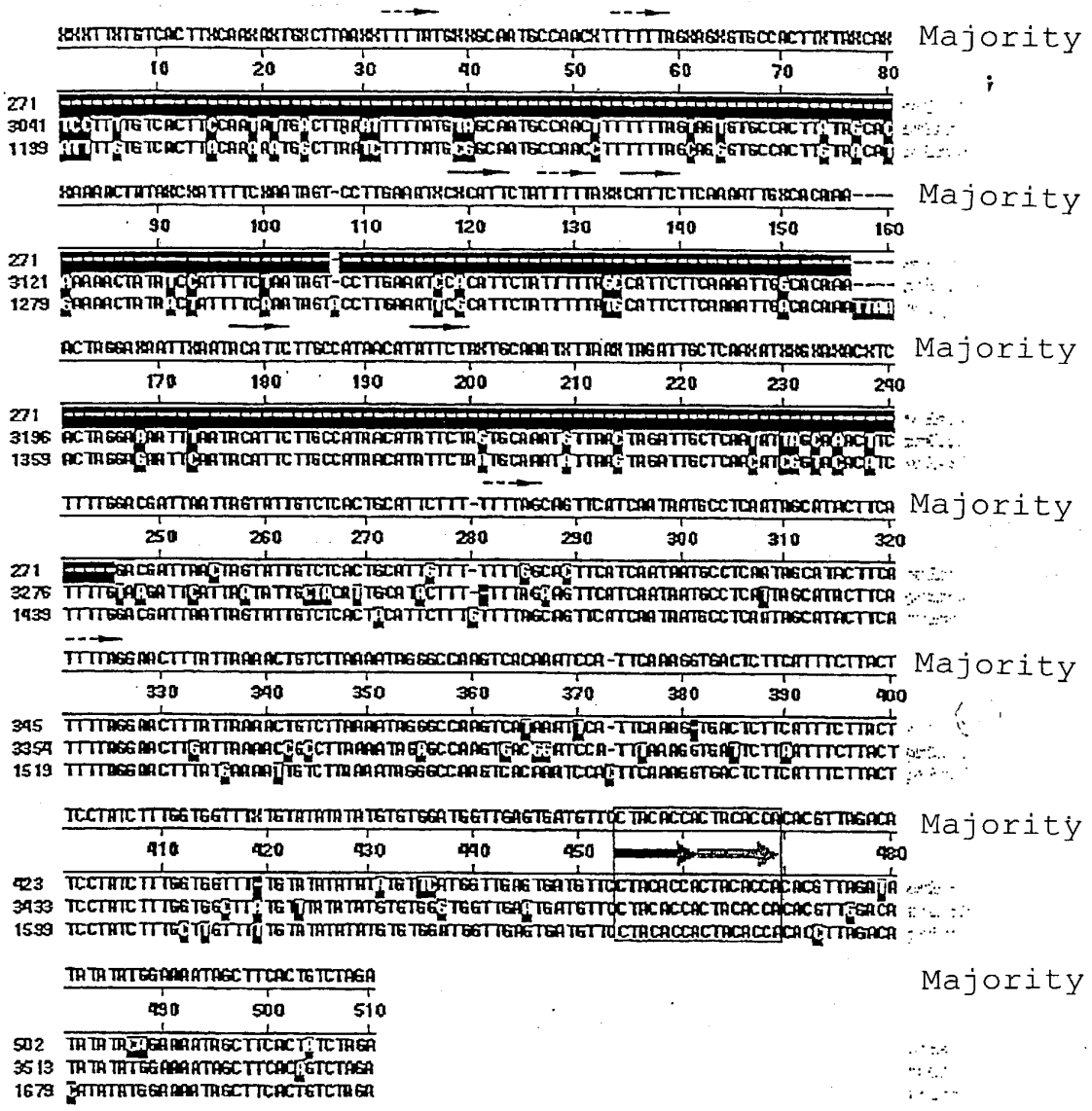
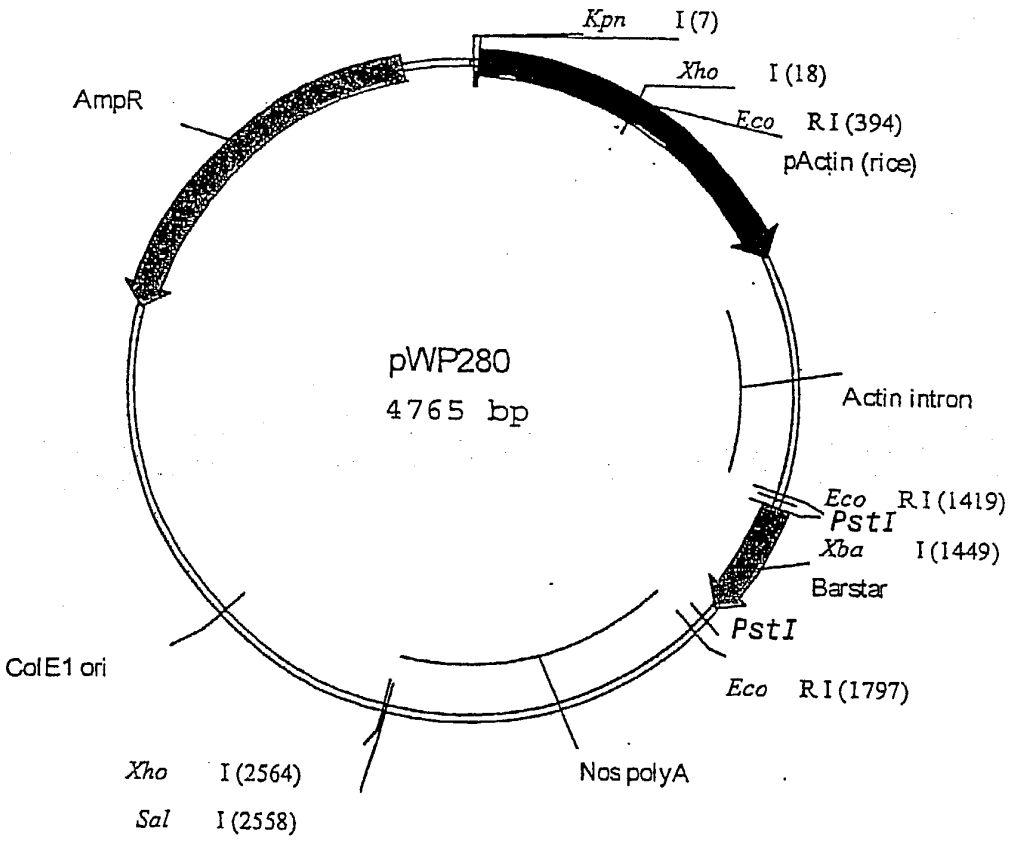


FIG.9

Repeat CTACACC in tandem

Repeat TTTTA Repeat ATTCT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Xho I (18)
Eco R I (394)
pActin (rice)
AmpR
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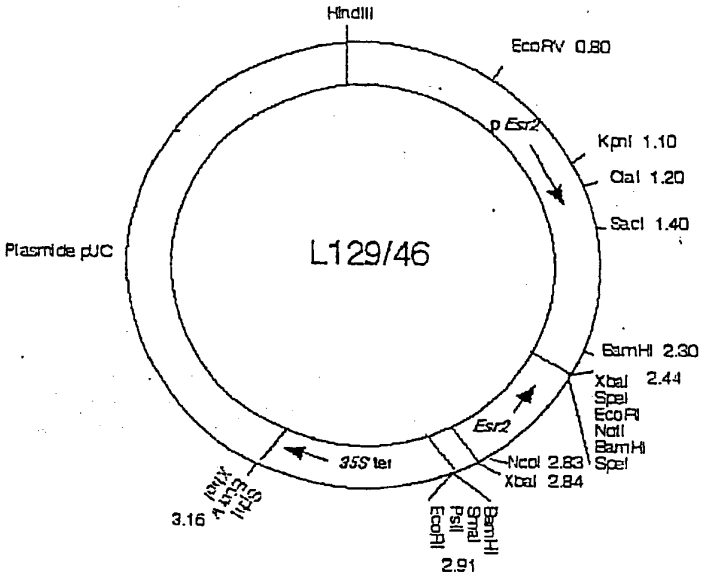
Actin intron
Eco R I (1419)
PstI
Xba I (1449)
Barstar
PstI
Eco R I (1797)
ColE1 ori
Xho I (2564)
Sal I (2558)
Nos polyA

FIG.10



Promoter or Es.z gene
Codified sequence for Esr2 gene
Terminator Ca MV 35 S

FIG.11

Ref. _____

COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PLANT SEED ENDOSPERM-SPECIFIC PROMOTERS

the specification of which: *(check one)*

REGULAR OR DESIGN APPLICATION

[ ] is attached hereto.

[ ] was filed on _____ as application Serial No. _____ and was amended on _____ (if applicable).

PCT FILED APPLICATION ENTERING NATIONAL STAGE

[X] was described and claimed in International application No. PCT/FR0002596 filed on 19/09/2000 and as amended on _____ (if any).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

PRIORITY CLAIM

I hereby claim foreign priority benefits under 35 USC 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN APPLICATION(S)

Country	Application Number	Date of Filing (day, month, year)	Priority Claimed
FRANCE	9912305	01/10/99	YES

(Complete this part only if this is a continuing application.)

I hereby claim the benefit under 35 USC 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of 35 USC 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application Serial No.) (Filing Date) (Status--patented, pending, abandoned)

POWER OF ATTORNEY

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from _____ as to any action to be taken in the Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

As a named inventor, I hereby appoint the registered patent attorneys represented by Customer No. **000466** to prosecute this application and transact all business in the Patent and Trademark Office connected therewith, including: **Robert J. PATCH, Reg. No. 17,355, Andrew J. PATCH, Reg. No. 32,925, Robert F. HARGEST, Reg. No. 25,590, Benoît CASTEL, Reg. No. 35,041, Eric JENSEN, Reg. No. 37,855, Thomas W. PERKINS, Reg. No. 33,027, and Roland E. LONG, Jr., Reg. No. 41,949,**

00466
PATENT TRADEMARK OFFICE

c/o YOUNG & THOMPSON,
Second Floor,
745 South 23rd Street,
Arlington, Virginia 22202.

Address all telephone calls to Young & Thompson at 703/521-2297. Telefax: 703/685-0573.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

Full name of sole or first inventor: Jean-François BONELLO
(given name, family name)

Inventor's signature _____ Date 25/03/02

Residence: 9 Rue J. Monet - 77600 BUSSY ST GEORGES - FRANCE Citizenship: French FRX

Post Office Address: The same as above

2-00

Full name of second joint inventor, if any: Peter ROGOWSKY
(given name, family name)

Inventor's signature _____ Date 25/03/02

Residence: 31 Rue André Bollier - 69007 LYON - FRANCE Citizenship: German FRX

Post Office Address: The same as above

3-00

Full name of third joint inventor, if any: Pascual PEREZ
(given name, family name)

Inventor's signature _____ Date 25/03/02

Residence: 17 Chemin de la Pradelle VARENNES - 63450 CHANONAT Citizenship: French FRX

Post Office Address: The same as above

Full name of fourth joint inventor:
(given name, family name)

Inventor's signature _____ Date _____

Residence: Citizenship:

LISTE DE SEQUENCES

<110> Biogemma

<120> Promoteurs spécifiques de l'albumen des graines de végétaux

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tatttttag ctattggatc gagcagttta gtaaaggtaa actacattta tctatcttca 540
agttgtattt tcccaccctt aaattatgaa agggagtaac gctccactcc aactgttgaa 600
agggaacaaa tttggtctcc ggactgattt cattggtggt tctctatttt ttaaaacaac 660
aaaaaaacat atttgttctc tgaaaattga taattaatta atcataaatt aggaaaaaaa 720
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tatagagtta taacatgaaa actatagcca ttttcaaata gtgccttgaa attcatttt 840
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taacatattc tagtccaaat gttaagtgga ttgctcaata tcattatact tcttttggac 960
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<211> 499
<212> ADN
<213> Zea mays

<400> 5
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tatttttagc cattcttcaa aattggcaca aaactaggaa aatttaatac attcttgcca 180
taacatattc tagtgcaaat gttaactaga ttgctcaata ttagcaaact tcttttgtaa 240
gattcattaa tattgctaca ttgcatactt ttttagaagt tcatcaataa tgcctcatta 300
gcatacttca ttttaggaac ttgattaaaa ccgccttaaa atagagccaa gtgacggatc 360
catttaaagg tgattcttaa tttcttactt cctatctttg gtggcttatg tttatatatg 420
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aatagcttca cagtctaga 499

<210> 6
<211> 507
<212> ADN
<213> Zea mays

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ctatttttat gcattcttca aaattgacac aaattaaact aggagaattc aatacattct 180
tgccataaca tattctaatg caaatattaa gtagattgct caacatcggt acacatcttt 240
tggacgatta attagtattg tctcactaca ttctttgttt tagcagttca tcaataatgc 300

ctcaatagca tacttcattt taggaacttt atgaaaattg tcttaaaata gggccaagtc 360
acaaatccac ttcaaaggtg actcttcatt tcttacttcc tatctttgct tgtttttgta 420
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<210> 7
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<212> ADN
<213> Zea mays

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rratycantt yaaagntgay tcttmatttc ttacttccta tctttgstkg yttwngtwta 180
tatatrtgtk srtggttgar tgatgttcct acaccactac accacacstt rgayayatat 240
ayrgaaaata gcttcacwrt ctaga 265

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<220>
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artificielle:oligonucléotide

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<210> 9
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artificielle:oligonucléotide

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<210> 10
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<210> 11

<211> 30
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<400> 17
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<400> 18
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<210> 19
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<210> 20
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<210> 21
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<400> 21
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